MECHANISMS OF FLOOD-TOLERANCE IN *RUMEX* SPECIES

Peter Laan

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een wetenschappelijke proeve op het gebied van de
natuurwetenschappen, in het bijzonder de biologie

PROEFSCHRIFT

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"Het water heeft namelijk, wanneer het voor bevloeiing wordt aangewend zo'n levenwekkend vermogen, dat het wanneer het de grond maar even bevochtigt, betere resultaten geeft dan ander water dat geruime tijd op de landen staat"
(opgetekend door Flavius Josefus, ca. AD 100)

"I'm looking at the river, but I'm thinking of the sea"
(Randy Newman, 'In Germany before the war')

Met dank aan allen die mij geholpen hebben!

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Front cover: *'Peter Pan'*
(from the novel by J.M. Barrie,
redrawn by John Slippens)

Back cover: *'Three penquins on ice'*

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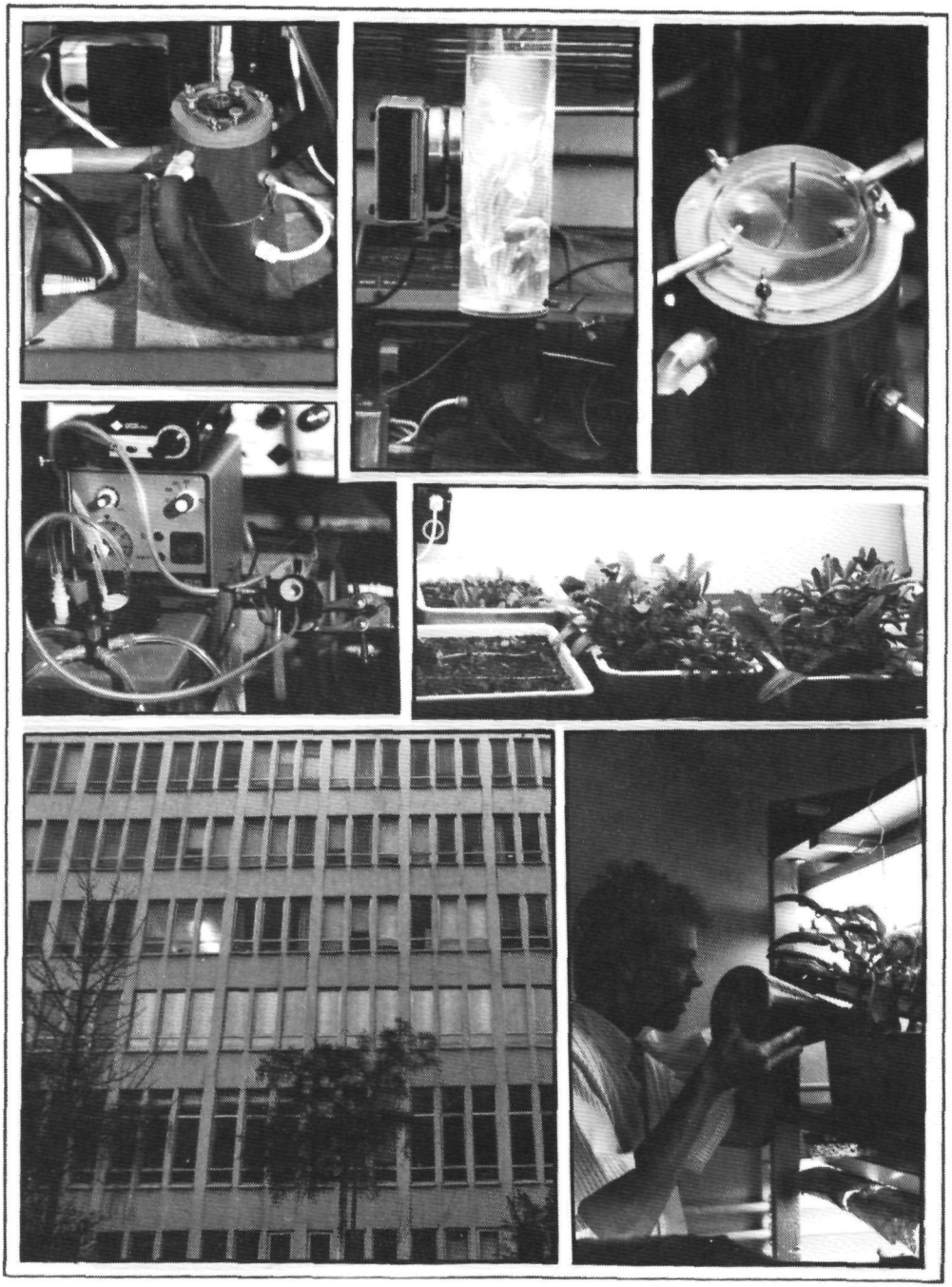

VOORWOORD

Wetenschap moet vooral spannend zijn. Vele ervaringen rijker kan ik na deze 4 ½ jarige periode inderdaad stellen dat hetgene we gedaan hebben inderdaad tot het laatste moment spannend is geweest. Hierdoor is mijn enthousiasme voor het beoefenen van wetenschap dan ook alleen maar toegenomen en hopelijk heeft dit zijn uitstraling naar anderen gehad, die in dit enthousiasme hebben kunnen delen. Gelijktijdig met spanning en enthousiasme moet wetenschap vooral gekenmerkt zijn door samenwerking in een sfeer van vertrouwen: gezamenlijk delen van voor- en tegenslagen, elkaar stimuleren en ideeën uitwisselen zijn onlosmakelijk verbonden met het goed en plezierig uitvoeren van wetenschappelijk onderzoek. Ik heb het geluk gehad de afgelopen jaren in dergelijke omstandigheden te mogen verkeren en een groot aantal mensen heeft dan ook bijgedragen aan de totstandkoming van dit proefschrift, want ik realiseer me dat me dit alleen nooit op deze manier gelukt zou zijn. Aan deze mensen wil ik hierbij mijn dank uitspreken. Dat betreft natuurlijk allereerst mijn promotoren. Dank aan prof. Cees Blom, voor het vertrouwen dat hij in mij gesteld heeft om samen met mij, als eerste promovendus, het afdelingswerk vorm te geven en mij daarna de vrijheid heeft gelaten een groot deel van het onderzoek zelf in te mogen vullen. Ook prof. Hans Lambers zeg ik hierbij dank voor zijn bijdragen op meer fysiologisch gebied, met name in het initiële stadium van dit promotieonderzoek, waarbij ik een aantal experimenten op zijn laboratorium heb mogen uitvoeren. Van zijn deskundigheid en zakelijke aanpak heb ik veel geleerd.

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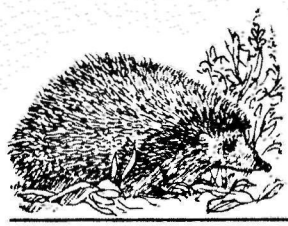
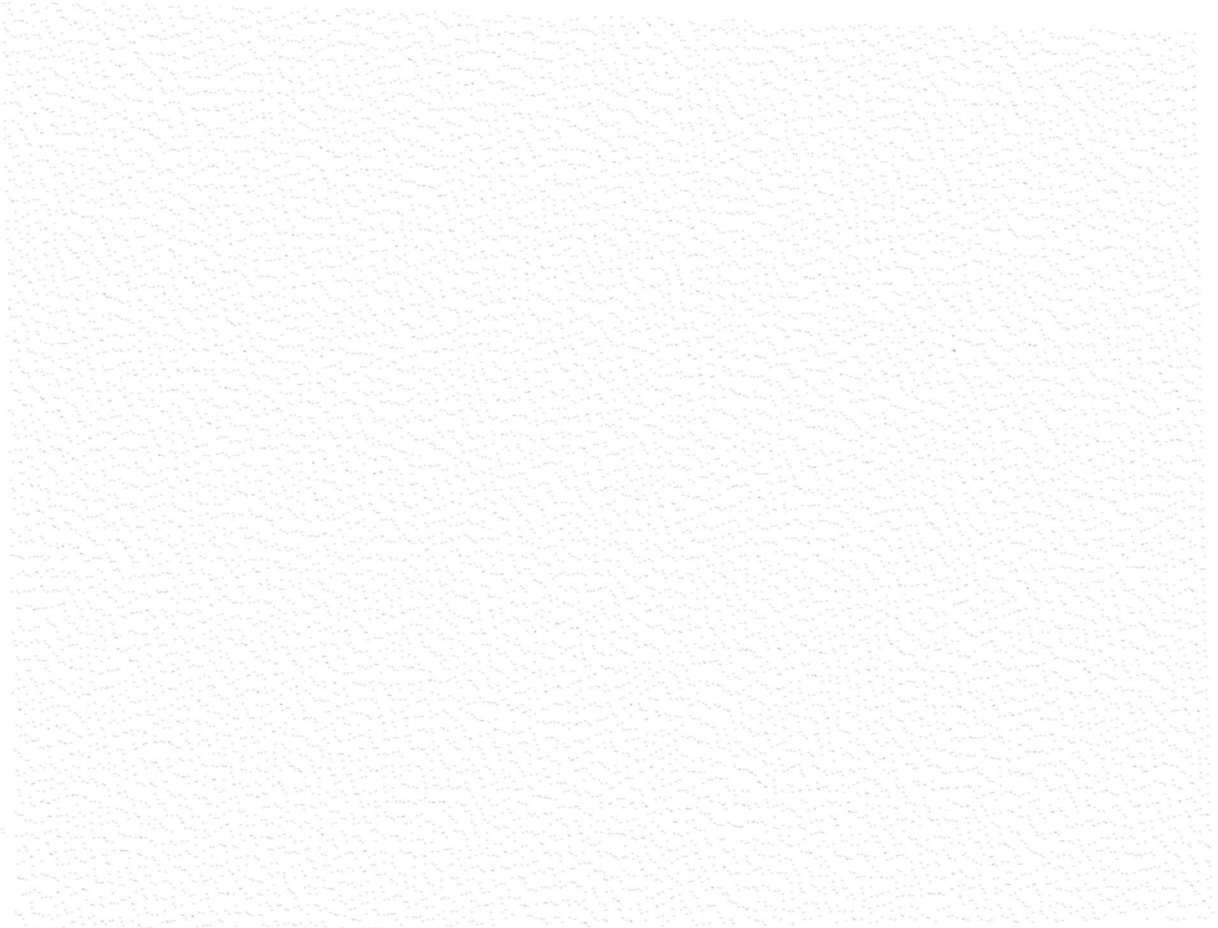
CHAPTER 1 General Introduction





Flooding of arable land is a well-known phenomenon, typical for coastal areas and river deltas and is especially important in countries in South and South-East Asia. In some of these countries it forms one of the main problems for food supply, as is the case in Bangladesh and North India. On the other hand, large-scale artificial flooding of land is of extraordinary importance for the food supply of South-East Asia, i.e. for the growth of deep-water rice. But also in the eastern regions of the USA, flooding can have an enormous impact on the yield of crops (Vantoai *et al.* 1987).

The river ecosystem in the Netherlands

In the Netherlands infrequent flooding of land takes place from early spring to late summer during the last decades in the lower parts of the Rhine delta (Van de Steeg 1984; Brock *et al.* 1987; Blom 1990). Snow melt and rainfall normally lead to high-water peaks of the river Rhine, that either inundate the river foreland during winter or can be buffered by small embankments. Due to an improved drainage of water in the higher parts, however, high-water peaks exceed heights, that can no longer be buffered by embankments in the lower part of the system, i.e. in the Netherlands. As a consequence, large areas of the river forelands are inundated for longer or shorter periods from early spring until August. Time of the year the floodings take place, height of the flood-level, duration and frequency are unpredictable (Blom 1990; Blom *et al.* 1990).

Since many river forelands in the Netherlands are characterized by elevation differences, and thus to the extent to which they are inundated, these floodings have had their impact on plant species distribution, and a typical plant zonation can often be distinguished (Van de Steeg 1984). Amongst others, *Rumex* forms one of the plant genera that is abundantly represented in the river ecosystem. It consists of typical 'wetland' species (e.g. *R. hydrolapathum* and *R. conglomeratus),* typical 'dryland' species (e.g. *R. acetosa* and *R. thyrsiflorus)* and of intermediate ones (e.g. *R. crispus* and

R. obtusifolius). Most of these *Rumex* species occur in the highly dynamic river forelands, there being partly zoned according to their different degrees of flood-tolerance. The genus furthermore contains species with different life-histories; although the larger part is covered by perennial species, biannuals and annuals are found as well (e.g. *Rumex palustris* and *R. maritimus).* Most of this thesis deals with three representatives of the *Rumex* zonation, being: **a)** *R. thyrsiflorus,* a perennial 'dryland' species, occurring on higher elevation levels in the river ecosystem on dry, sandy haylands and meadows. According to its elevation level, this species is proposed to be flood-intolerant, **b)** *R. crispus,* a very common perennial species from lower elevated and rather wet loamy soils. It has a very wide amplitude in the river ecosystem and, although its position is intermediate in the zonation, *R. crispus* must be refferred to as highly flood-tolerant towards both short- and long-term flooding, **c)** *R. maritimus,* a biannual or annual species, abundantly occurring on low elevated and frequently drained muds. Together with *R. palustris, R. maritimus* dominates at the lowest elevation levels in the river ecosystem and is highly flood-tolerant.

All *Rumex* species under study are characterized by the formation of a new root system upon flooding or anaerobiosis (Fig. 1). Under drained conditions of the soil or, under aerobic conditions in hydroculture, primarily formed roots, originating from the tap-root, function to enable growth and ion uptake. Under situations of soil-flooding or anaerobiosis in hydroculture, new roots are formed, which can easily be distinguished from the primarily formed ones (Fig. 1). This newly formed root system apparently counteracts the adverse effects brought about by soil-anaerobiosis, for especially flood-tolerant species develop an extended network of these roots within a short period (see Chapter 2). Thus, upon anaerobic conditions the *Rumex* root system is characterized by the unique combination of consisting of two separate ones and it is proposed (Chapters 2 and 7) that the balance between these two root systems will therefore play a major role in the flood-tolerance of the species.

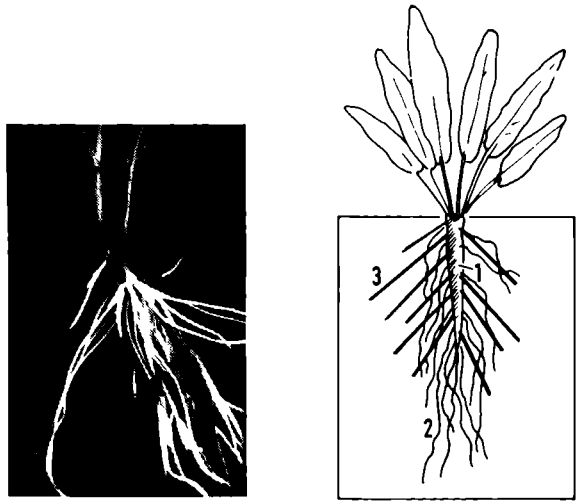
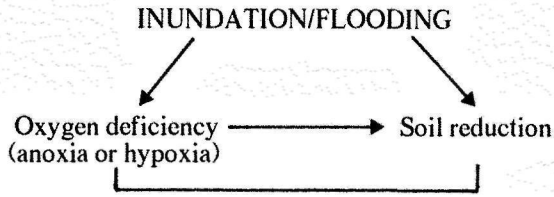


Figure 1 The *Rumex* root system: view of the root system of *R. maritimus* after 3 weeks of flooding in sand **(left)** and schematic representation of the build up after flooding or, after subjection to anaerobic conditions in hydroculture **(right)**. Root type 1, tap-root; 2, primary lateral roots; 3, newly formed lateral roots, developed in response to anaerobic conditions

Adverse effects of flooding on plant growth

The primary problem flooding brings about is the exhaustion of oxygen from the soil, predominantly caused by respiratory activity of aerobic soil micro-organisms; this process can be completed within a few hours (Yoshida & Tadano 1978; Ponnamperuma 1984). The resulting lack of oxygen and the concomitant soil reducion may give rise to a wide range of injurious effects on plants, which have been extensively reviewed (e.g. Armstrong 1979; Crawford 1982; Drew 1983; Jackson & Drew 1984). The main problems can be summarized in four major groups (Table 1): **a)** Effects on the nutrient balance of the shoot, either a deficiency of macro-nutrients or the accumulation of potential phytotoxins, **b)** The impact of flooding on energy metabolism. The primary problem for the plant is the decrease in ATP yield in roots, but the occurrence of toxic end products of fermentation processes can also form part of a disturbed metabolism, **c)** A hormonal imbalance. Ethylene, as a gaseous plant hormone has been studied most extensively, but the role of

Table 1 Potential injurious effects of flooding on plants



PLANT INJURY

A) *Plant ion content*
Deficiency of macro-nutrients (especially N and K)
-growth inhibition
-leaf senescence
Accumulation of micro-nutrients
-toxicity (Fe, Mn, S)

B) *Energy metabolism* (ATP shortage)
-Growth inhibition
-Decrease in energy-dependent ion uptake and transport
-Toxicity of endproducts of fermentation (ethanol, lactate)

C) *Hormonal imbalance* (especially ethylene, gibberellins and cytokinins)
-growth inhibition
-leaf senescence
-epinasty
-stomatal closure and reduced photosynthesis

D) *Water deficiency* (temporary effect)
-wilting of leaves,
-stomatal closure and reduced photosynthesis

gibberellins and cytokinins seems to be at least as important, **d)** Water deficiency, which often leads to wilting of leaves, but in most cases seems to be temporarily.

Acclimation and adaptation mechanisms

Plants have to develop resistance mechanisms to be able to cope with the adverse effects of flooding. Since root cells of all plants, including 'wetland' and aquatic plants, are obligate aerobic (Vartapetian *et al.* 1978; Armstrong 1979), the most outstanding adaptations on the longer term are those, which relieve the oxygen deficiency of the root

system. With only a few exceptions, those species that are capable of performing or inducing this 'avoidance' strategy, can cope with prolonged periods of anaerobiosis and can be referred to as flood-tolerant.

The most common anatomical acclimation undoubtly is the development of a longitudinally connected system of lacunae in the root cortex, which is referred to as aerenchyma (Arber 1920; Sculthorpe 1967; Armstrong 1979; Smirnoff & Crawford 1983). Aerenchyma can be formed lysigenously (by lysis of cells in the cortex), or schizogenously (by cell separation), but in all cases reduces diffusive resistances for gases. The development of aerenchyma provides the plant with a continuous connection between root and shoot, which allows the diffusion of gases. Oxygen, either from the air or from photosynthesis, diffuses from shoot to roots and the primary need of the root system for oxygen can be partly or completely satisfied, even at low oxygen concentrations in the root environment This 'internal aeration' is therefore thought to be the main basis of long-term flood-tolerance (Armstrong 1979; Armstrong & Beckett 1985; Ap Rees *et al.* 1987; Brändle 1990; Chapter 4, this thesis), which may enable sustained growth and ion uptake by flooded roots.

Next to anatomical and morphological adaptations, metabolic acclimation can help to overcome or modify the oxygen stress for a relatively short time. Metabolic acclimation mechanisms are easily induced, but the sole use of them will never lead to long-term flood-tolerance. Plant species which mainly rely on metabolic adaptation mechanisms can therefore only be distinguished between the very intolerant and the less intolerant ones. The most important response of plant cells under hypoxia or anoxia is the acceleration of glycolysis to gain more ATP per time unit. This 'Pasteur effect' in general is accompanied by the induction of pyruvate decarboxylase and alcohol dehydrogenase, two key enzymes of anaerobic fermentation reactions. It is interesting to notice that especially flood-intolerant species have a high ADH-induction and/or 'Pasteur effect' (Crawford 1967, 1978, 1982). Although a relatively high ATP-level can be maintained for a couple of hours, a shortage of respirable sugars is likely to occur (Jackson & Drew 1984). Thus,

for these species, ADH-induction and the occurrence of a 'Pasteur effect' cannot be recognized as an (efficient) adaptation mechanism, but may rather be referred to as a 'stress' reaction. In addition, since metabolic adaptations are acting at a cellular level, an induction is merely related to the total number of cells being in a hypoxic or anoxic state. Because flood-intolerant species often lack the ability to relieve the oxygen stress, more cells will be oxygen deficient and thus ADH-induction will be strong.

This does not mean that the 'Pasteur effect' and ADH-induction are of minor importance in flood-tolerance. Induction is found in both flood-tolerant and -intolerant plant species. For flood-intolerant species, metabolic adaptation can be useful to overcome short-term flooding periods, or they can be additionally important, next to other adaptation mechanisms. But also for flood-tolerant species they can serve an important function. This can be the case in a number of situations: **1)** when transient flooding takes place and morphological adaptation has not yet had the time to develop, metabolic adaptations can help to overcome the first period of anaerobiosis, **2)** in situations of partial flooding, a 'Pasteur effect' can be of considerable importance during the night period. Shoot dark respiration can be quite high and oxygen deficiency is likely to occur in the roots, in spite of the capability of oxygen transport (Waters *et al.* 1989), **3)** notwithstanding a strong development of aerenchyma in the root cortex, oxygen deficiency in other vital parts of the root may occur. This is especially true for the stelar zone, which can easily become anoxic, due to early woodyness of the endodermis (Armstrong 1979; Armstrong & Beckett 1985, 1987; Armstrong 1989), **4)** when aerenchyma formation takes some time to develop after flooding, as is typical for the major part of plant species that develop a lysigenous aerenchyma, the oxygen requirements of the compact root apex can not be fullfilled and metabolic adaptation can help to maintain a relatively high ATP-level (Armstrong 1979; Jackson & Drew 1984).

Next to the induction of a 'Pasteur effect' and ADH-induction, it has been reported that nitrate may be useful in anaerobic metabolism. Nitrate, as an alternative electron

acceptor, can withdraw the surplus of NADH-equivalents (Garcia-Novo & Crawford 1973; Bertani *et al.* 1987), in this way enabling glycolytic reactions to continue. Although it is often shown that nitrate can partly alleviate the adverse effects of flooding, there is no consensus on the mechanisms by which this is reached (see Chapter 9).

Unlike morphological or metabolic adaptation mechanisms, adaptations on the life-cycle level can be of significant importance in the development of resistance to stress conditions like flooding. The best known example is the situation in which plants, confronted with severe stress like total submergence, change to a state of 'early dormancy'. Such a dormancy is characterized by a slowing down of plant metabolism and can help to overcome periods of complete oxygen deficiency, hereby relying on anaerobic metabolism and fermentation. It necessitates some sugar storage capacity and is therefore most probably found among perennial plant species.

Aims and outline of the present thesis

The ecophysiological approach of this thesis is mainly focussed on an understanding of mechanisms underlying the differences in flood-tolerance of *Rumex* species. Techniques and methods used to achieve this main aim cover both greenhouse and hydroculture experiments; in all cases an imitation of the real situation under field conditions was pursued, which implied a reduction of the number of variables under study. In many cases, simulation using cuvettes in which only one or two factors can be varied suited this scope best; next to this, greenhouse experiments in which the plants were grown on a natural substrate, were used as a 'feed-back' to field conditions. This combined approach allows an interpretation of the results to the field situation in terms of 'potential factors, that may play a role in the differential flood-tolerance of the plants'.

In the early phases of this project it appeared that above all, oxygen supply to the root system would be most important for the differential flooding resistance of the *Rumex* species. It is mainly therefore that a large part of this thesis deals with oxygen,

either as a primary subject of study (Chapters 4 and 5) or as a derived one (Chapters 6, 7 and 8).

In Chapters 2 and 3 the basal characteristics of changes in root morphology and concomitant root anatomy of several *Rumex* species upon soil-flooded conditions are described and related to the different position of the species in the elevation zonation sequence. The functional significance of aerenchyma formation or a high root porosity in terms of oxygen supply to the root cells is treated in Chapters 4 and 5. The ultimate consequences of the observed differences in oxygen supply of the root systems for survival are elucidated in Chapter 6 and for growth and iron exclusion in Chapters 7 and 8. In Chapter 9 the importance of metabolic adaptation for the flood-tolerance of three of the *Rumex* species is treated. The results are discussed in the context of the 'strategies' used by the species to cope with low oxygen levels.

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ROOT MORPHOLOGY, AERENCHYMA FORMATION AND OXYGEN LOSS

CHAPTER 2 Root morphology and aerenchyma formation as indicators of the flood-tolerance of *Rumex* species

with M.J. Berrevoets, S. Lythe, W. Armstrong & C.W.P.M. Blom
Journal of Ecology (1989) **77**: 693-703 (with permission)

CHAPTER 3 The relative roles of internal aeration, radial oxygen losses, iron exclusion and nutrient balances in flood-tolerance of *Rumex* species

with A. Smolders, C.W.P.M. Blom & W. Armstrong
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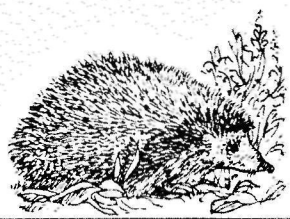

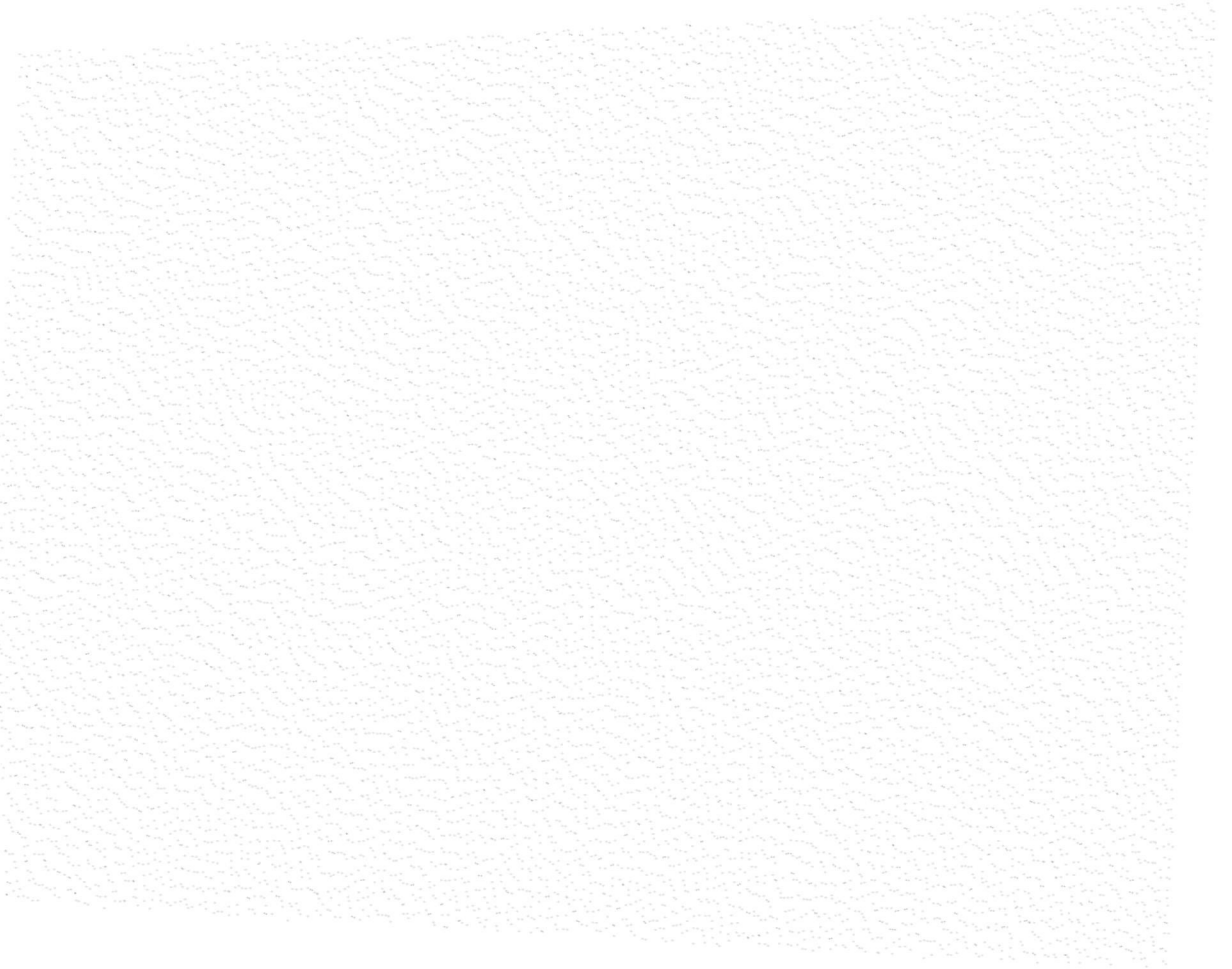

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ROOT MORPHOLOGY AND AERENCHYMA FORMATION AS INDICATORS OF THE FLOOD-TOLERANCE OF *RUMEX* SPECIES

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SUMMARY

(1) *Rumex* species are zoned along a gradient of elevation in the river ecosystem in The Netherlands.

(2) Plants of *R. thyrsiflorus*, *R. acetosa*, *R. obtusifolius*, *R. crispus*, *R. conglomeratus* and *R. maritimus* were flooded to identify and quantify any relevant adaptive features and to test whether their distribution might be caused by a differential response to flooding in the growing season.

(3) Most *Rumex* species have a tap-root from which the laterals originate. As a response to flooding, new laterals are formed.

(4) The number, place of origin, growth direction and formation rate of new laterals differed between the species.

(5) The number and formation rate of new roots were associated with the elevational distribution of the species: as a response to flooding, low-elevation species formed more new roots, and faster, than high-elevation species.

(6) The high-elevation species had root porosity values lower than 10%; the intermediate- and low-elevation species had values higher than 10%.

(7) Schizogenous aerenchyma was constitutively formed by the low-elevation and flood-tolerant *R. maritimus*, and not by the high-elevation and flood-intolerant species *R. thyrsiflorus*. In the intermediate-elevation species *R. crispus* it was induced in stagnant hypoxic solution cultures.

(8) The results indicate that aerenchyma formation is closely connected with the growth rate of new roots. It appears that development of aerenchyma in the new roots is the main determinant in the flood-tolerance of *Rumex* species.

INTRODUCTION

Flooding induces a number of responses in plant roots, of which aerenchyma formation is one of the most obviously adaptive (Arber 1920; Sculthorpe 1967; Armstrong 1979; Konings & Verschuren 1980; Crawford 1982; Jackson & Drew 1984; Justin & Armstrong 1987). Aerenchyma is formed either by some cell wall separation and cell collapse (lysigeny) or by cell separation without collapse (schizogeny). Both forms result in larger longitudinal channels in the root cortex. This structure enhances the diffusion of atmospheric or photosynthetic oxygen via, or from, the shoot to the roots so that aerobic respiration and growth can be maintained (Armstrong & Gaynard 1976; Lambers, Steingröver & Smakman 1978; Armstrong 1979; Armstrong & Webb 1985; Drew, Saglio & Pradet 1985).

Changes in root morphology also occur after flooding and this is true for both wetland and non-wetland species. There may be: (i) an increase in branching of the roots (Vose

1962; Geisler 1965); (ii) the development of new, adventitious roots (Bergman 1920; Kramer 1951; Alberda 1953; Arikado & Adachi 1955; Jackson 1955; Drew, Jackson & Gifford 1979; Wenkert, Fausey & Waters 1981; Hook 1984); and (iii) superficial rooting (Alberda 1953; Armstrong & Boatman 1967; Sheikh & Rutter 1969; Schat 1984).

In most cases newly formed roots are more porous than roots of the primary root system (Arikado & Adachi 1955; Luxmoore & Stolzy 1969; Schat 1984; Justin & Armstrong 1987) and development of new, porous roots is thought to be beneficial to the whole root system and thus to plant growth and development (Jackson 1955; Stanley, Kaspar & Taylor 1980). Development of such newly formed roots may be especially advantageous in situations of transient flooding or in plant species which are unable to form aerenchyma in the primary root system.

In order to test whether such features might accord with plant distribution within the genus *Rumex*, a number of species was subjected to various flooding regimes and the responses compared. Most of the species used occur as a zonal pattern along an elevational gradient in the river ecosystem in The Netherlands; this is predominantly caused by transient flooding in the growing season (Van de Steeg 1984). At the lowest elevations *R. maritimus* L. dominates. At slightly higher elevations *R. crispus* L. and *R. obtusifolius* L. are found, whilst *R. acetosa* L. and *R. thyrsiflorus* Fingerh. occur above high flood levels. *R. conglomeratus* Murr. occurs in periodically wet, low elevation sites behind the river flood-banks.

All the species except *R. acetosa* produce a tap-root from which the laterals originate and, in response to flooding, secondarily formed laterals are developed to different extents. In *R. acetosa* new roots are formed on the older primary roots or near the root–shoot junction.

In this study, the morphology and gas-space development of the root systems are described and the differences considered in relation to the differential flood-tolerance and distribution of the species.

MATERIALS AND METHODS

Plant growth

Seeds of *Rumex thyrsiflorus*, *R. acetosa*, *R. obtusifolius*, *R. crispus*, *R. conglomeratus* and *R. maritimus* were collected from natural populations in the river area near Nijmegen (The Netherlands).

Sand-culture

Seeds of all species were sown in Petri dishes on wet Whatman filter paper and left to germinate at 25 °C (day), 15 °C (night), 16 h light at 60–100 μmol m^{-2} s^{-1} PAR, 8 h dark. After a week a batch of seedlings was transferred to river sand (organic matter content $0{\cdot}5 \pm 0{\cdot}1\%$) in 3·3-litre pots (height 18 cm), and allowed to grow for three to four weeks in a glasshouse (*c.* 19 °C, relative humidity 70%, 16 h light at minimum 100–150 μmol m^{-2} s^{-1} PAR, 8 h dark). Half of these plants were then subjected to soil flooding by placing the pots in 50-litre plastic containers, which were slowly filled with quarter-strength Hoagland's solution (Hoagland & Arnon 1950) until the water level was 1–2 cm above the soil surface. Black polyethylene grains (low density grains, British Petroleum, Grangemouth, U.K.) floating on the water suppressed growth of algae. The water level was checked daily. Control, non-waterlogged plants were watered every two or three days with quarter-strength Hoagland's solution. At various times during the treatment,

drained and flooded plants, eight per treatment, were harvested at random and carefully washed on a sieve to remove the sandy soil. The roots were washed twice to remove the remainder of the adhering particles, and could then be used to determine root length, distribution of laterals over the tap-root, and root porosity.

Another batch of seedlings was transferred to vertical PVC-tubes (76 cm long, diameter 7·5 cm) filled with river sand. A metal grid covered with a piece of nylon was mounted on the bottom of the tubes allowing free contact between the nutrient solution and the substrate. The plants were allowed to grow for seven weeks, after which the tubes were flooded to 1–2 cm above the soil surface with quarter-strength Hoagland's solution. The water was subsequently maintained at this original level. After three weeks of flooding, the plants were harvested by carefully pushing out the sand core complete with root system. Root morphology was described and maximal depth of the different root types was recorded.

Hydroculture

Five-week-old plants of *R. thyrsiflorus*, *R. crispus* and *R. maritimus* were grown either in aerated hydroculture, using quarter-strength Hoagland's solution (aerated plants), or in perspex containers with quarter-strength Hoagland's solution in anaerobic 0·05% agar (stagnant plants). Stagnant plants developed new lateral roots with a length of 4–8 cm within one to two weeks. Primary lateral roots of the aerated plants and newly formed laterals of the stagnant plants were used for examination of root anatomy.

Root porosity by pycnometry

After seven, fourteen and twenty-one days of flooding, three plants were harvested at random and the root systems carefully washed on a sieve. The apical 20–30 cm of the different types of new roots were cut off with a razor-blade. Root porosity was measured with a pycnometer (Jensen *et al.* 1969); gas was not removed by maceration, but by evacuation on a freeze-dryer.

Root length

Total root length of secondarily formed roots (i.e. roots formed after flooding) was measured with a ruler or, when length exceeded 1 m, with a root length scanner (Comair, accuracy *c.* 0·1 m).

Distribution of laterals

Plants which had been flooded for three weeks were separated into shoots and roots. Tap-roots were divided into segments and, when necessary, the shoot was divided into segments of about 1 cm. Secondarily formed laterals were counted per segment; they were not woody and were thus distinguishable from primary laterals.

Root anatomy

The apical first centimetres of the primary lateral roots and 1-cm segments from the apical 4–5 cm of the newly formed lateral roots were fixed in 3% (w/v) $KMnO_4$ (30 min), washed and stored in distilled water at 3 °C. The segments were dehydrated in an acetone series (30, 50, 70, 90 and 100%, 10 min each step) and, after rinsing twice in 100% acetone, placed in propylene oxide for 45 min. The root segments were then embedded in a resin mixture (Epon 812 : Araldite 6005 : DDSA = 3 : 3 : 8; Frances Allen method, modified from Coffey, Palevitz & Allen 1972) by gradual replacement of the propylene oxide by the resin

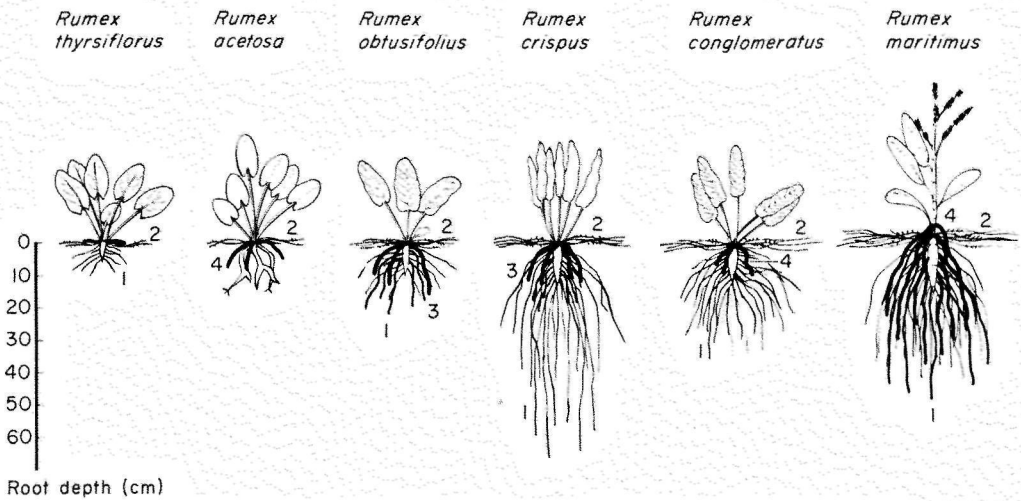

FIG. 1. Root morphology and root depth of *Rumex* species after three weeks of flooding in river-sand (age of the plants at the start of the experiment six weeks; original lateral root system not shown). Root type: 1, downward-growing laterals; 2, horizontally-growing laterals; 3, thick downward-growing laterals; 4, adventitious roots.

mixture. The resin was allowed to polymerize for 24 h at 70 °C. Sections of 1 μm from halfway along the blocks were cut with a Cambridge ultra-microtome. The sections were stained with 1% (v/v) toluidine blue in 1% (w/v) borax and photographed on a Zeiss photomicroscope. Gas-space cross-sectional area as a proportion of total root cross-sectional area was determined using an area-meter (MOP-system, Kontron, GMBH).

RESULTS

Changes in root morphology after flooding

The species showed considerable differences in root morphology after three weeks of flooding in river-sand (Fig. 1). In *R. thyrsiflorus* the primary laterals died within a week and few new roots were formed after flooding: some very thin and unbranched horizontally growing laterals, growing on or just below the water surface, and some downwardly growing laterals which soon ceased growth. After three weeks the apical 3–5 cm of the longest new downward-growing laterals had a transparent appearance, indicating that they were fully watersoaked and had ceased to function.

In *R. acetosa* the primary laterals, as in *R. thyrsiflorus*, died within one or two weeks and even fewer new laterals were formed after flooding, most of which were short and concentrated in the superficial zone. Here too, newly-formed laterals ceased growth and the tips died after some time, but before this occurred, new superficially growing roots were formed. Most of these roots originated above the root–shoot junction (adventitious roots), were very thick and unbranched, and also remained short (type 4; Fig. 1).

In *R. obtusifolius*, *R. crispus* and *R. conglomeratus* the primary laterals did not die when flooded, but the root system of each of these species was extended considerably by the formation of different types of new laterals. In particular, large numbers of horizontally growing laterals were formed on or just below the water surface, and in *R. conglomeratus* these formed a compact root mat after three weeks (type 2; Fig. 1). All three species also

formed adventitious roots and, in addition, *R. obtusifolius* developed some very thick and unbranched downwardly growing laterals (type 3).

R. maritimus showed the most extensive root outgrowth in response to flooding. Not only was there a rapid development of large numbers of downward and horizontally growing laterals (types 1 and 2), but also of adventitious roots, formed on the nodes of the flower stalk, which penetrated the soil within only one week (type 4; Fig. 1). Most of the primary laterals continued to grow.

Course of events leading to changed root morphology and differential responses to flooding

Four types of response, which more or less characterize the different species, could be distinguished.

Type 1

In *R. thyrsiflorus* only a few new roots (on average twenty-nine per plant) were more or less uniformly distributed over the tap-root (Fig. 2a); the same type of response was found in *R. acetosa*, which also developed few new roots. Growth of these new roots was slow and hardly significant (Fig. 2c). After two weeks of flooding the accumulated total length of new roots, with very low porosity (3–6%, Fig. 2b), was 3·0 m and 3·5 m in *R. acetosa* and *R. thyrsiflorus*, respectively, less than 10% of the total length at the start of the treatment (Fig. 2c).

Type 2

In *R. crispus* and *R. obtusifolius* more new laterals developed than in *R. thyrsiflorus* or *R. acetosa* (forty-seven and twenty-eight per plant, respectively), and these too were more or less uniformly distributed over the tap-root. Due to the formation of aerenchyma, however, high porosity values were reached (15–22%). As a consequence, growth of the new roots was significant within a week. This resulted in an accumulated total length of 14 m and 16 m in *R. obtusifolius* and *R. crispus*, respectively, after two weeks; this was 26% and 32% of the total length of the roots at the start of the treatment (Fig. 2c).

Type 3

In *R. conglomeratus* many new laterals were formed (on average 108 per plant), most of them originating at the tap-root apex (Fig. 2a). Root porosity was high (exceeding 20%) and the final accumulated length of newly formed roots was 43 m after two weeks of flooding; hence, the extent to which the original root system was replaced was very high (44%, Fig. 2c). This high final rate of growth was not only caused by the large number of new roots in combination with a high root porosity, but also by regrowth of the original root system and the formation of secondary laterals on the old roots.

Type 4

R. maritimus developed as many new roots as *R. conglomeratus* (115 per plant) but the distribution pattern was opposite: most of the new roots were formed on the shoot and the basal parts of the tap root (58% of total in segments 1–3; Fig. 2a). The initial growth rate of the new roots, with a very high porosity (25–35%, Fig. 2b), was high and an acceleration of growth could be observed after outgrowth of the root primordia of the adventitious roots on the first stem node (i.e. after five days). This resulted in a final accumulated total length of 24 m, which equals 23% of the initial root length (Fig. 2c).

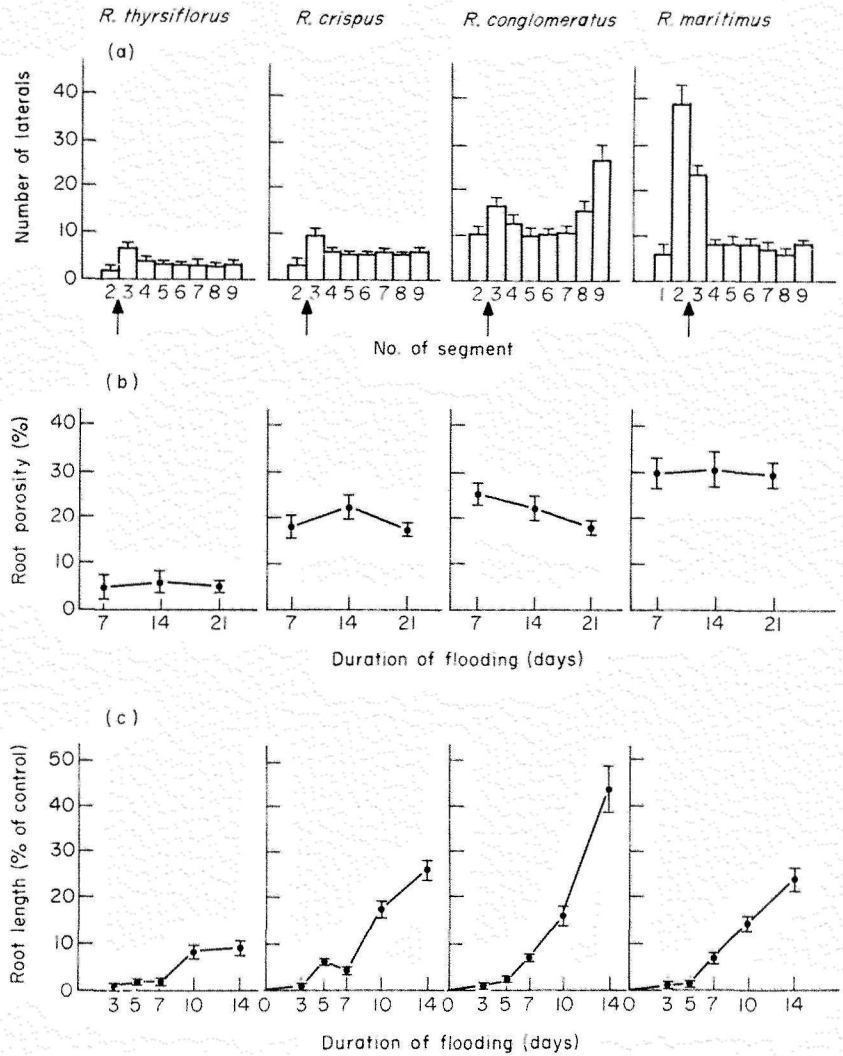

FIG. 2. Effects of flooding on the root systems of some *Rumex* species growing in river sand in a glasshouse. (a) Position of origin of newly formed roots. Segments 1 and 2: 1-cm root segments above the root–shoot junction; segments 3–9: 1-cm root segments below the root–shoot junction; arrows indicate the position of the root–shoot junction; values are means + 1 S.E. ($n = 8$); (b) root porosity of newly formed lateral roots: values are means + 1 S.E. ($n = 3$); (c) extension rate of primary root system, i.e. the total length of the newly formed root system as a percentage of the length of the primary root system at the start of the flooding treatment; values are means + 1 S.E. ($n = 8$); initial lengths of primary root systems were: *R. thyrsiflorus* 38·2 m; *R. crispus* 61·5 m; *R. conglomeratus* 99·3 m; *R. maritimus* 104·6 m. All plants were twelve weeks old at the start of the treatment.

Root anatomy

An explanation for the observed differences between the *Rumex* species might be found in a differential ability to form aerenchyma. Such differences were already suggested by the root porosity data (Fig. 2).

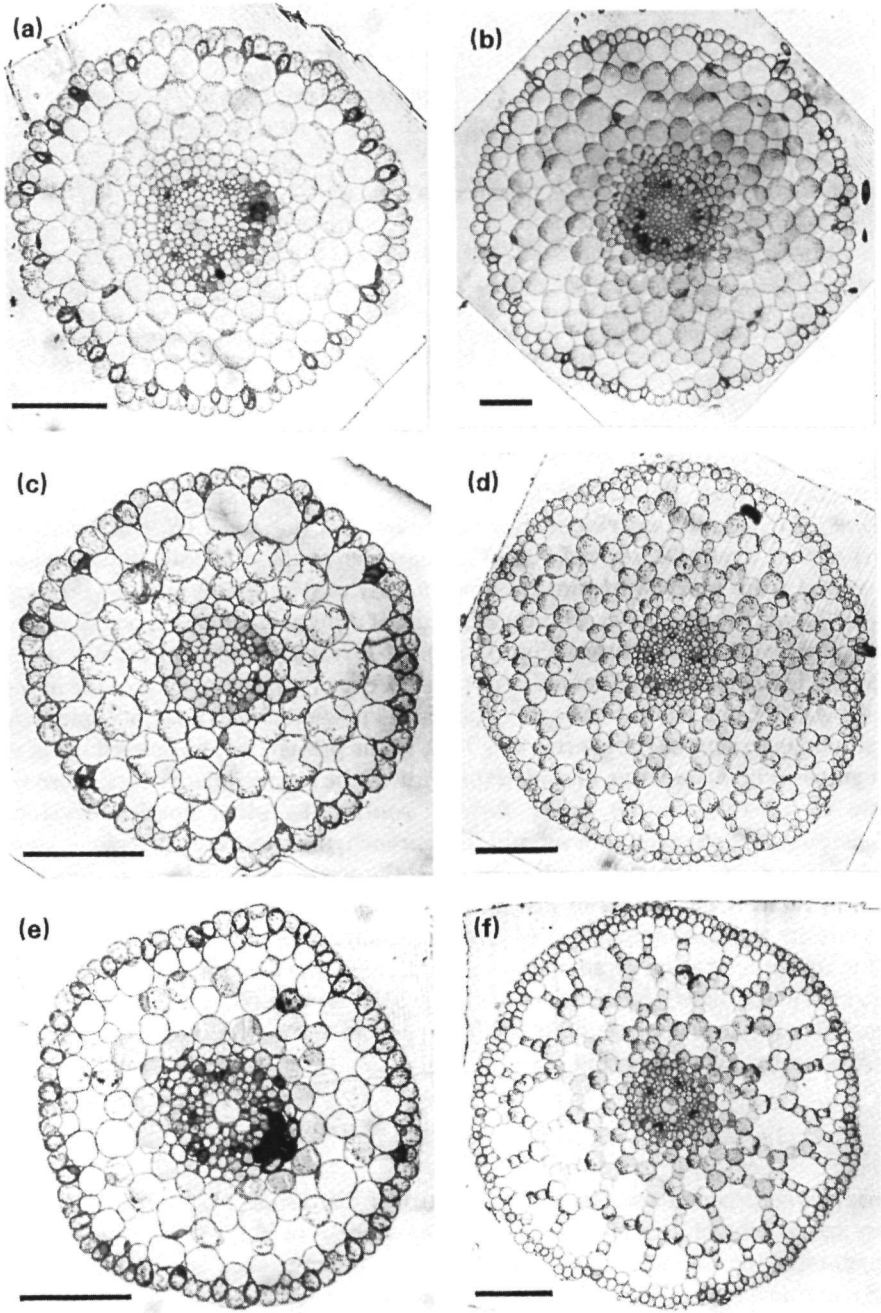

FIG. 3. Cross-sections at 0·5 cm behind the apex of lateral roots of *Rumex thyrsiflorus* (a), (b), *R. crispus* (c), (d) and *R. maritimus* (e), (f), formed under aerobic (a), (c), (e) and stagnant anaerobic (b), (d), (f) hydroculture conditions. Bars represent 100 μm.

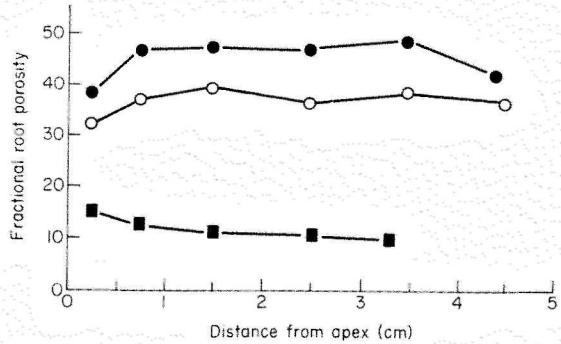

FIG. 4. Fractional root porosity (FRP) along newly formed roots of *Rumex thyrsiflorus* (■), *R. crispus* (○) and *R. maritimus* (●). (FRP is the percentage of the root-cross sectional area of the whole root occupied by gas-space.)

Plants precultured in aerated hydroculture and then transferred to a stagnant anaerobic agar solution developed new laterals within one week (*R. maritimus* and *R. crispus*) or two weeks (*R. thyrsiflorus*). The diameter of these laterals (2 cm behind the apex) varied from $0{\cdot}62 \pm 0{\cdot}04$ mm (*R. thyrsiflorus*) to $0{\cdot}72 \pm 0{\cdot}02$ mm (*R. crispus*) and $0{\cdot}84 \pm 0{\cdot}04$ mm (*R. maritimus*). There were clearcut differences in gas-space development between the investigated species (Figs 3 & 4). *R. thyrsiflorus* was apparently unable to form aerenchyma in anaerobic conditions (Fig. 3), even at the basal ends of the new roots (Fig. 4). At 0·5 cm behind the root apex the cross-sectional area occupied by the intercellular spaces in the root cortex was 7·7% in the primary roots (percentage of whole root segment, Fig. 3) and, due to some expansion of the intercellular spaces, there was an increase to 10–15% in the newly formed roots. The other species developed a schizogenous aerenchyma ('honeycomb'-like aerenchyma structure). *R. crispus* was non-aerenchymatous in aerobic conditions and the cross-sectional area occupied by intercellular spaces was similar to that in *R. thyrsiflorus* (7·8% at 0·5 cm behind the apex; Fig. 3) but the stagnant anaerobic conditions caused a four- to five-fold increase to 30–40% due to the formation of aerenchyma. In *R. maritimus*, aerenchyma was formed in both primary and newly formed laterals (Fig. 3). This was reflected in the high cross-sectional area of the primary laterals (15·7% at 0·5 cm behind the apex, Fig. 3) increasing to 40–50% in the new roots (Fig. 4).

DISCUSSION

In those *Rumex* species studied, their elevational distribution in the river ecosystem appears to be related to the type and degree of new root formation and gas-space development. After two weeks of flooding, the accumulated total length of the new root system was much higher in *R. obtusifolius*, *R. crispus*, *R. conglomeratus* and *R. maritimus* than in *R. acetosa* and *R. thyrsiflorus* (Fig. 2). These new laterals are supposed to take over the function of the old root system (Hook 1984; Jackson & Drew 1984) and in the low- and intermediate-elevation species more than 20% of the old root system was replaced by the new one after ten to fourteen days (Fig. 2). Formation of this new root system was probably beneficial to the root system as a whole, for regrowth of the old root system and/

or development of new laterals on the older roots occurred after ten to fourteen days of flooding in sand in *R. obtusifolius*, *R. crispus*, *R. conglomeratus* and *R. maritimus*. The number of horizontally growing roots, floating on or just below the water surface (Fig. 1) and as high in porosity as the downward growing roots, seemed to be correlated with the elevational distribution of the species: the lower the elevation, the higher the total length of horizontally growing laterals. Not much is known about their function, but the fact that they occur on or just below the water surface suggests that they serve functions other than improvement of the oxygen status of the root system, e.g. ion uptake.

Growth rate of the new root system depended on both number and porosity of the roots. Although the total number of new roots formed in *R. obtusifolius* and *R. crispus* was not much higher than in *R. acetosa* and *R. thyrsiflorus*, their high growth rate and concomitant replacement of the old root system was apparently a result of aerenchyma formation (Fig. 3). In both *R. maritimus* and *R. conglomeratus* a large number of new roots with high porosity was formed. As a consequence, the old root system was extended considerably in both species after two weeks (Fig. 2). *R. maritimus*, however, developed twice as much length of new root as *R. conglomeratus* after three days (1·23 m *v.* 0·60 m); together with the opposite distribution pattern (Fig. 2), this may help to explain the differences in habitat of the two species: *R. conglomeratus* predominantly occurs behind the main dam in stagnant waters, while *R. maritimus* is frequently confronted with fluctuating water levels.

Root growth is very sensitive to the oxygen concentration near the root apex (Armstrong 1979) and roots of *R. thyrsiflorus*, *R. crispus* and *R. maritimus* of comparable length differed significantly in their apical oxygen pressure, correlated with porosity (Laan *et al.* 1989). Development and outgrowth of new laterals were well correlated with the capability of a species to form aerenchyma (Figs 3 & 4). The high porosity values of the primary roots of *R. maritimus* (Fig. 3) could also help to explain the observation that these roots keep growing when flooded. Aerenchyma formation often distinguishes flood-tolerant from flood-intolerant plants; Smirnoff & Crawford (1983) distinguished between tolerant and intolerant species at 10% porosity; Justin & Armstrong (1987) concluded that most non-wetland species have porosities lower than 7%. In both *R. thyrsiflorus* and *R. acetosa* porosity values were generally lower than 7–10%; in all other species the values generally exceeded 10% (Fig. 2). It should be noted, however, that a small number of wetland species do not form aerenchyma, and survive the wetland habitat by means of shallow rooting (Justin & Armstrong 1987). This phenomenon may also be important for *Rumex* species (Fig. 1).

Compared to the porosity values obtained from the anatomical preparations (Fig. 4), the porosity values of new roots of *R. acetosa* and *R. thyrsiflorus*, determined by pycnometry (Fig. 2), were very low (3–6%). Such low values might be due to a proportion of dead roots in the samples and to the use of older (newly formed) root material, which contains more woody tissue and, in addition, shows progressive branching.

Fractional root porosity values showed that the area occupied by intercellular spaces was already 7–8% under aerobic conditions in both *R. thyrsiflorus* and *R. crispus* (Fig. 3). However, only *R. crispus* formed aerenchyma in the new roots (Figs 3 & 4). This suggests that in these *Rumex* species aerenchyma formation is of more importance in determining flood-tolerance than porosity *per se*. As a wetland-species, *R. maritimus* develops aerenchyma constitutively (i.e. both in the primary and the new roots) but an increase in porosity as high as in *R. crispus* resulted in very high porosity values in the new roots (Figs 3 & 4).

A study of the morphology and the anatomy of the tap-roots showed clearcut differences between the species. All developed primary lateral roots in three rows on the tap-root. The roots, formed in response to flooding, grew close to the primary roots, except in *R. thyrsiflorus*, in which these new roots were distributed at random over the tap-root. This random growth habit could be a disadvantage when it results in laterals far from aerenchyma and other transport-channels, so that oxygen transport via the tap-root to the laterals is hampered. On the other hand, in *R. maritimus* three big lacunae could be observed in the basal parts of the tap-root, and more than 50% of the new roots originated from the basal 2 cm of the tap-root and from the shoot (Fig. 2). This anatomy correlates well with the very high rate of oxygen transport from shoot to roots under hypoxic conditions (internal aeration) found in this species (P. Laan and B. W. Veen, unpublished data).

The results obtained from the sand cultures should be interpreted with care in relation to the actual flood-tolerance of the species in the river ecosystem. Although it is stated that the oxygen concentration is low in flooded sand culture (Smirnoff & Crawford 1983), the reducing power of clay substrates, as found in the river ecosystem, can be greater than that of sand. However, experiments performed on clay substrate revealed responses of the *Rumex* species to flooding comparable to those reported here, with the exception that there were no clearcut differences between *R. crispus* and *R. maritimus* in their growth rate and biomass production upon flooding (Laan *et al.* 1989). In that case, factors other than root morphology and aerenchyma formation alone apparently determine the elevational distribution of these species.

In conclusion, in those *Rumex* species studied, aerenchyma formation is closely connected with the growth rate of new roots upon flooding, and the total number of lateral roots, plus their distribution over the tap-root, at least partly explain the differential responses towards transient flooding of the intermediate- and low-elevation species. To explain the distribution of these species in the river ecosystem fully, however, other aspects will need to be considered, e.g. the age of the plants, the flowering stage at the onset of flooding, and the responses to total submergence.

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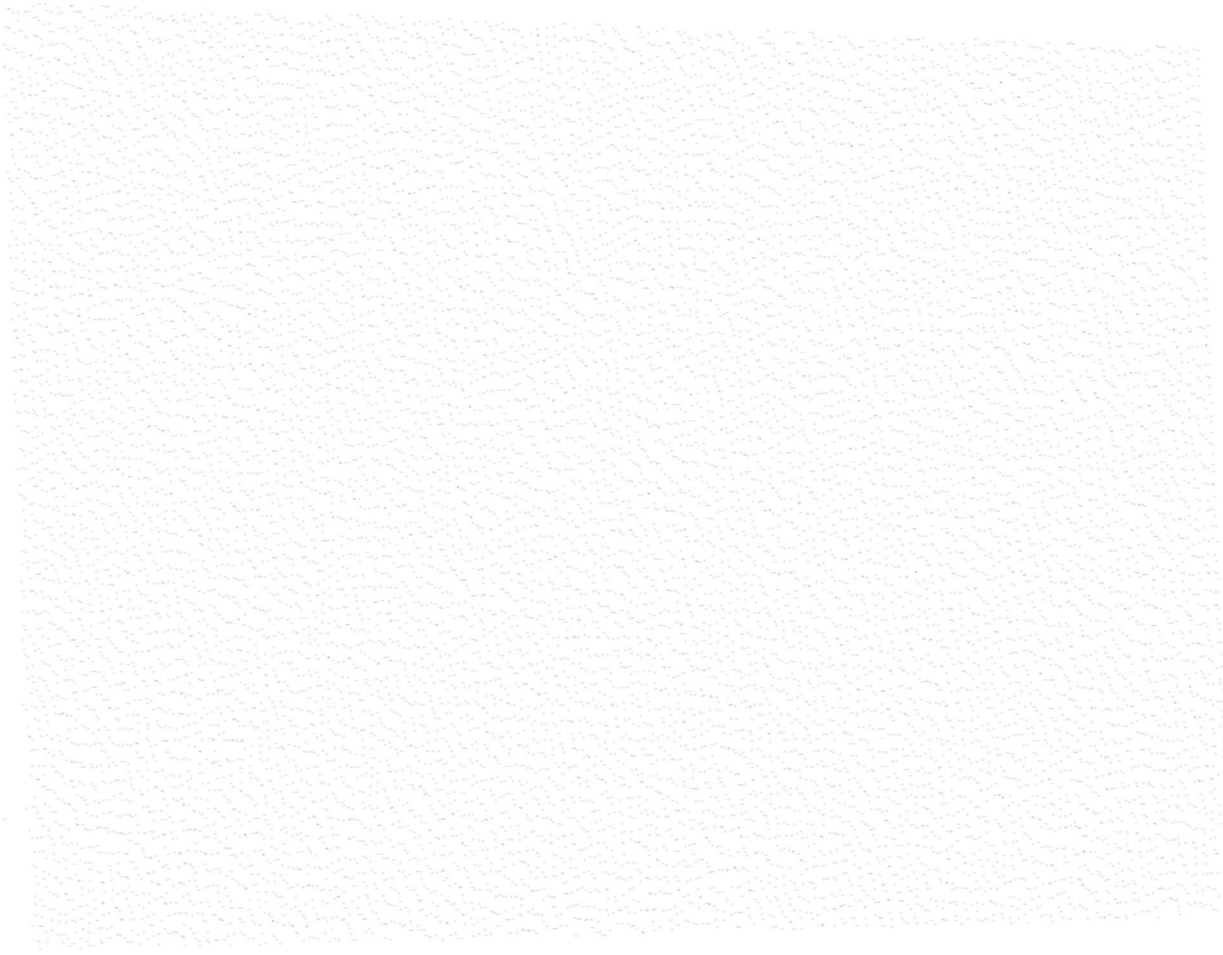

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The relative roles of internal aeration, radial oxygen losses, iron exclusion and nutrient balances in flood-tolerance of *Rumex* species

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SUMMARY

Radial oxygen losses (ROL) from the roots of three *Rumex* species, that occur in the river ecosystem in The Netherlands and show a differential response towards flooding, were compared. Oxygen loss from whole root systems was demonstrated and the ROL of single roots was quantified. Radial oxygen losses were higher in the flood-tolerant *R. maritimus* and *R. crispus* than in the intolerant *R. thyrsiflorus*. In all species oxygen loss occurred over the whole root surface between the base and the apex, but the rates differed as well as root wall permeabilities to oxygen. High oxygen losses in *R. maritimus* and *R. crispus* were correlated with high internal oxygen pressures near the root apex, consistent with prolonged root growth under anaerobic conditions in these species. On a flooded clay soil, the more tolerant species showed soil penetration and iron oxidation to greater depths, but all species developed an iron plaque on the roots. Shoot iron content was highest in the flood-tolerant *R. maritimus*. Upon flooding of the flood-intolerant *R. thyrsiflorus*, however, there was a substantial decrease in shoot dry weight and tissue nutrient levels. This was attributed to restricted root development rather than to iron toxicity.

Key-words: flood-tolerance, iron toxicity, nutrient stress, oxygen loss, *Rumex*.

INTRODUCTION

The process of rhizosphere oxidation by plant roots has been known for very many years (Molisch 1887; Groenewege 1922). This oxidation and the release of oxygen *per se* are now described as important characteristics of flood-tolerance (van Raalte 1944; Yoshida & Tadano 1978; Armstrong 1967, 1971, 1979; Ottow *et al.* 1982). It is generally accepted that the primary beneficial effect of radial oxygen loss (ROL) is the fact that potential soil toxins, such as reduced iron, manganese and hydrogen sulphide, which can increase to toxic concentrations under anaerobic conditions, can be immobilized or detoxified (Bartlett 1961; Armstrong 1967, 1978, 1979; Armstrong & Boatman 1967; Jones & Etherington 1970; van Breemen & Moormann 1978; Ottow *et al.* 1982). Most striking and of considerable importance is the accumulation of ferrous iron in the soil solution

(Ponnamperuma 1972). The subsequent oxidation creates clearly visible iron oxide and hydroxide precipitations on the roots (Groenewege 1922; Molisch 1926; van Raalte 1944; Armstrong 1967). A high and efficient ROL is said to slow down iron uptake, and to help prevent iron toxicity in the shoot (Tanaka *et al.* 1966; Martin 1968; Armstrong 1978, 1979; van Breemen & Moormann 1978; Yoshida & Tadano 1978; Ottow *et al.* 1982; Rozema *et al.* 1985; Iremonger & Kelly 1988). It has also been suggested, however, that iron plaque formation and iron oxide precipitations in the rhizosphere might hinder the uptake of essential nutrients (Howeler 1973; Benckiser *et al.* 1984; Wheeler *et al.* 1985; Otte *et al.* 1989), and in this respect ROL could be a disadvantage for the nutrient supply of the plant. In addition some doubts have been expressed recently concerning the significance of 'direct' iron toxicity as a determinant of flood tolerance and/or species distribution (Benckiser *et al.* 1984; Etherington 1984; Schat 1984).

Oxygen loss from roots can be demonstrated or quantified by several techniques. Dye techniques can easily be performed to show the location of oxygen loss in whole root systems (e.g. van Raalte 1941; Armstrong 1967; Trolldenier 1988). The ROL of single roots can be quantified accurately by polarographic measurement; the so-called 'cylindrical platinum electrode' method (Armstrong 1964, 1979) seems to be the most suitable. With this method ROL can be quantified at different positions along the root. It also allows the calculation of the internal oxygen concentration near the root apex, which is the final outcome of such root characteristics as the magnitude and distribution of respiratory demand, porosity and root wall permeability, and is closely correlated to root growth (Armstrong 1979).

In this study we compare the ROL of three *Rumex* species, which occur in the river ecosystem in The Netherlands and show a differential response towards flooding (Laan *et al.* 1989). One of the most obvious responses of these plants is the formation of a new root system upon flooding; the new roots of the tolerant species *R. maritimus* and *R. crispus* contain aerenchyma, but those of the intolerant *R. thyrsiflorus* do not. The significance of aerenchyma formation, and concomitant oxygen loss for the differential flood-tolerance of the species, was tested by growth of the plants in a flooded clay substrate. Attention was paid to the possible role of iron toxicity as a determinant for the flood-tolerance of the *Rumex* species, and the effects of flooding on the nutrient status were examined.

MATERIALS AND METHODS

Root oxidation of leuco-methylene blue

Plant growth. Seeds of *Rumex thyrsiflorus* Fingerh., *R. crispus* L. and *R. martitimus* L., collected from natural populations in the river area near Nijmegen (The Netherlands), were sown in trays containing black polyethylene grains (low density grains, BP Grangemouth, UK), which were totally submerged in 25% full strength modified Hoagland's solution (Hoagland & Arnon 1950). The trays were covered with a glass plate and placed in a germination cell for 1–2 weeks (25°C (day), 15°C (night); 16 h fluorescent light (Philips TL 33) at 60 μmol $m^{-2} s^{-1}$, 8 h dark). After germination the young plants and young tillers of rice plants (*Oryza sativa* L.) were allowed to grow for 2–3 weeks in a growth room (temperature 25°C, RH 70%; 16 h fluorescent light at 200 μmol $m^{-2} s^{-1}$ (LI-COR photometer), 8 h dark). They were then carefully transplanted to containers (13×10^{-3} m^3), filled with 25% Hoagland's solution and allowed to grow for another week. Some of these young plants were grown aerobically by bubbling air through the nutrient solution, while

another batch was transferred to containers, filled with a stagnant anaerobic 0·1% (w. agar in 25% Hoagland's solution; the latter were kept there until they had formed new lateral (*Rumex*), or adventitious (rice) roots. The water level of all the containers was maintained at the original level and nutrient solutions were changed twice a week. The pH of the unadjusted nutrient solution was 5·5 and remained constant during the experiment.

Experimental assembly. Plants were carefully transferred to a flat glass box (40 × 30 × 1 cm) with the shoot protruding through a small recess at the side of the box. The root system was spread over the box and the location of the separate roots was fixed with small glass rods (diameter 3 mm). The box was then closed by carefully sealing the upper glass plate with vaseline, while the recess containing the lower shoot parts was sealed with plasticine. The inner part of the box was flushed with nitrogen gas for 15 min through tubing placed inside the box before closure. During this nitrogen flush a leuco-methylene blue solution (25 mg l^{-1}), reduced via titration with sodium dithionite and maintained under anaerobic conditions by a nitrogen flow, was added to the box through a glass union fixed to the upper glass plate. The union was then closed and the nitrogen flow stopped. From this starting point, oxidation of leuco-methylene blue by roots of comparable length was followed by photographing the box in a time series.

Quantification of radial oxygen loss with the 'cylindrical platinum electrode' method

Plant growth. Seeds of all three *Rumex* species were sown on vermiculite in a greenhouse (temperature 15–27°C; 16 h light at 60–100 μmol m^{-2} s^{-1}, 8 h dark). After germination and growth for 2 weeks, the plants were transferred to the modified 25% Hoagland's solution, and allowed to grow for another 5–6 weeks in a growth room (temperature 23°C, 16 h light at 150 μmol $m^{-2}s^{-1}$, 8 h dark). They were then transferred to a 25% Hoagland's 0·05% (w:v) anaerobic agar solution and within 1 (*R. crispus* and *R. maritimus*) or 2 (*R. thyrsiflorus*) weeks new laterals had developed with a length of 3–10 cm.

Experimental assembly and determination of radial oxygen loss (ROL). A plant was placed in a rectangular perspex vessel, which was completely filled with 0·05% (w:v) anaerobic agar solution (at least 24 h bubbling with oxygen-free nitrogen). The shoot base was sealed with wet cotton wool in a drilled rubber stopper. A cylindrical platinum cathode ($h = 5·0$ mm, diameter 2·25 mm) was drawn into position to ensleeve a newly formed lateral, and two small holes at the edge of the rubber stopper facilitated the positioning of the electrode at 0·2 cm from the root tip using a bicycle spoke. Saturated Ag/AgCl half-cells were used as anodes. Polarograms and determination of the ROL at a different position along the root were performed as described by Armstrong (1979) and Webb & Armstrong (1983). After determining a ROL profile, the position and length of the lateral on the tap-root were recorded. The diameter of the lateral was measured at the same positions where the ROL was determined using a travelling Vernier microscope (accuracy 0·01 mm, Precision Tools & Instruments, UK).

From the ROL data the root surface oxygen concentrations in the apical region were calculated according to Armstrong (1979). In this apical region, where root wall permeability is usually high, these root surface concentrations give a close approximation of the internal cortical gas-phase oxygen concentrations (Armstrong & Wright 1975; Armstrong & Webb 1985).

Growth of plants on a clay substrate

Experimental assembly. Plants germinated and pregrown for 2 weeks (as described in 'Root Oxidation of Leuco-Methylene Blue'), were transferred to vertical PVC tubes (diameter 12 cm, height 40 cm) and filled with a 1:1 (v:v) clay:sand mixture [river sand and clay from a beet-field (pH (H_2O) 6·9; weight percentage organic matter 5·4 ± 0·1)] which was enriched with some homogeneously distributed plant material. A PVC bottom containing four holes (diameter 6 mm) allowed free contact between the outer environment and the substrate. The plants were allowed to grow for 4 weeks in a greenhouse (*c.* 19°C, RH 70%, 16 h light, consisting of daylight filtered by greenhouse glass (120–1000 μmol $m^{-2} s^{-1}$) and supplementary light (high-pressure sodium lamp, Osram Vialox, 200 μmol $m^{-2} s^{-1}$) when light intensity decreased to values lower than 200 μmol $m^{-2} s^{-1}$; 8 h dark), after which half were flooded with tap-water to 2 cm above the soil surface. The other plants were used as controls; these tubes were checked daily and, when necessary, watered to maintain the original water content (drainage). During the flooding treatment the redox potential was recorded at different depths and the soil solution was sampled at the same depths to determine the 'free'-iron concentration.

After 4 weeks of flooding the plants were harvested by carefully pushing out the soil core complete with root system. The depth of oxidation of the soil, as indicated by its brownish colour, and clearly distinguishable from the reduced black soil, was recorded. Shoots were cut off and fresh weight and dry weight (24 h, 70°C) were determined; the root system was carefully washed out in a white container; the maximal root penetration and location of iron plaques and so-called micropedotubuli (Brewer 1964) were recorded during removal of the soil. Representative, randomly chosen living roots were sampled and used for the determination of iron content of the plaques and of the total amount of extracellular iron.

Redox potential measurements. Platinum wire electrodes were placed at different depths. The readings were stable after 1 or 2 days and these redox potentials were recorded (Pt cathode, saturated Ag/AgCl-anode, $E_O = 199$ mV).

Free iron concentration in the soil solution. Several times during the flooding treatment the soil solution was sampled at different depths with 2-ml pipettes. A piece of cotton-wool was pushed into the tip to prevent the entry of clay particles. The free-iron concentration was determined by adding 1 ml anaerobic 1 mM 2,2'-bipyridyl solution to 1-ml samples of soil solution, and after vigorous shaking A_{520} was determined (1 mM $Fe[bipyridyl]_3$ = 8·650). A few grains of sodium dithionite were then added to the samples and again the A_{520} was determined. When the difference between the absorbances exceeded 10%, the data were not taken into account, because this indicated a contamination of the samples with clay particles. The method did not allow sampling of the drained (control) soil.

Iron content of plaque on the roots. The apical 10–15 cm of the roots of the drained plants were very carefully extracted, and the roots of the flooded plants were separated into segments from the reduced and from the oxidized soil layers. One-gram fresh roots of drained plants and of root segments from the oxidized soil layers and 0·25 g of root segments from reduced soil layers were rinsed in demineralized water and the total amount of extracellular iron was determined after Bienfait *et al.* (1984); because of the expectedly high iron content, 4·5 instead of a 1·5 mM bipyridyl solution was used.

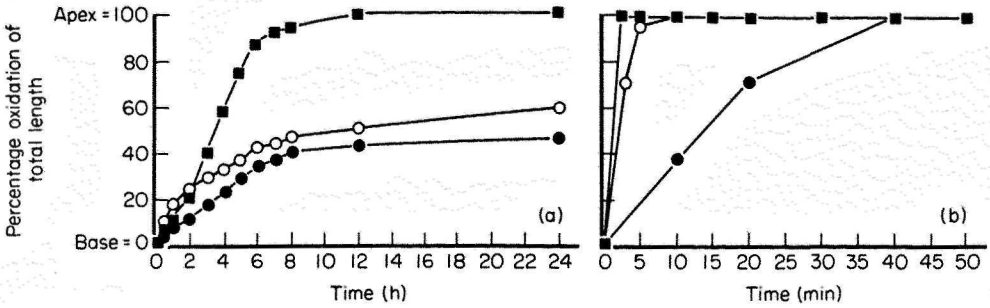

Fig. 1. Time course of readily perceived oxidation of leuco-methylene blue as blue coloration rate of primary (a) and newly formed (b) lateral roots of *Rumex thyrsiflorus* (●), *R. crispus* (○) and *R. maritimus* (■). Data as a percentage coloration of the total length of the root; base = 0%, apex = 100%; means of four replicates. Mean length of primary roots ± SD, *R. thyrsiflorus* 19·3 ± 4·4, *R. crispus* 22·0 ± 7·1, *R. maritimus* 24·4 ± 2·5 cm: newly formed roots: *R. thyrsiflorus* 5·0 ± 0·6, *R. crispus* 12·8 ± 2·5, *R. maritimus* 12·5 ± 2·0 cm.

Foliar nutrient levels. All dry leaves of the shoot system were ground in a sample mill (Tecator Cyclotec 1093) and broken down in a mixture of H_2SO_4 and salicylic acid (100 ml conc. H_2SO_4 in 18 ml H_2O + 6 g salicylic acid; overnight at room temperature for 1 h at 180°C). The destruction was completed by adding 400 µl 30% H_2O_2 (v:v), raising the temperature to 230°C (10 min), cooling down, adding another 400 µl H_2O_2, raising the temperature, etc. until the samples were colourless. Samples were analysed for calcium, magnesium, phosphorus and potassium on an inductive-coupled-plasma emission spectrophotometer (ICP); total nitrogen content was determined colorimetrically (Hampson 1977).

The total iron content of the leaves was determined on 50–100 mg dry leaf material ashed at 650°C, using the method of Scott (1944).

RESULTS

Root oxidation of leuco-methylene blue

Blue coloration at the root periphery could be observed in all the roots under investigation, indicating the presence of an oxidizing agent at or within the root surface. The time course of colour development along the root was recorded to compare the efficiency of the gas transport systems. Clear-cut differences between both species and root types were observed. The primary laterals of *R. thyrsiflorus* and *R. crispus* showed a similar pattern: a slow progress of surface blue coloration from base to apex, with respective maxima of 58% and 46% of the total length after 24 h (Fig. 1a). In *R. maritimus* blue coloration also proceeded from base to apex, but took place much faster: after 12 h, all the roots were completely blue (100%, Fig. 1a).

In the newly formed laterals of all *Rumex* species, leuco-methylene blue oxidation was much faster than in the primary laterals. Here again, coloration took place from base to apex, and was slowest in the intolerant species *R. thyrsiflorus* (100% coloration after 42 min, Fig. 1b). The tolerant species *R. crispus* and *R. maritimus* showed a much higher oxidation rate: the surfaces were completely blue after 10 and 2·5 min respectively (Fig. 1b). We used rice as a reference plant to check the method used, and to compare its responses with earlier results (Armstrong 1967, 1971; Trolldenier 1988). In rice, the blue coloration took place even faster than in *R. maritimus* and was complete within 1·5 min

(data not shown). It was impossible to observe whether the coloration started at the apical or the basal ends of the roots.

In addition to blue coloration, halo-formation took place, i.e. oxidation of leuco-methylene blue at a distance from the root. This halo-formation was most clearly visible in the newly formed laterals of the three *Rumex* species and in rice, and is indicative of rapid oxygen loss from the roots. In the primary laterals of the *Rumex* species some halo-formation also took place; in *R. crispus* and *R. maritimus* this was very poor and only visible after a long time. In the primary laterals of *R. thyrsiflorus* halo-formation started much earlier, but was restricted to the most basal parts of the root (data not shown).

The newly formed laterals of the *Rumex* species all showed progressive halo extension from base to apex (Fig. 2). In *R. maritimus*, halo-formation took place immediately after the start of the experiment and extended to 71% of the total root after 12 h; in both other species halo-formation became visible much later (>2 h after the start, Fig. 2c). While after 12 h *R. crispus* had reached a value similar to that shown by *R. maritimus*, halo-formation remained very poor in *R. thyrsiflorus* (33% of total length, Fig. 2a), even though the length of the roots was three times shorter than in *R. crispus*. Halo-formation in rice proceeded from apex to base and was complete after 3 h (100%, Fig. 2d). In the oxidation course from apex to base, there was a small subapical zone which showed a delayed halo-formation in all investigated roots.

Radial oxygen loss from single roots. Oxygen loss from single newly formed roots was quantified along the root with the cylindrical Pt-electrode method (Armstrong 1979). Because the amount of oxygen lost from lateral roots appeared to be determined both by the length of the lateral and by the originating point from the tap-root, the ROL profiles are presented as a function of the total apparent diffusion path length, i.e. the distance between the base of the tap-root and the originating point of the lateral on the tap-root plus the distance from the originating point of the lateral to the electrode (Fig. 3).

Roots of comparable length of *R. maritimus* and *R. crispus* showed much higher oxygen losses than those of *R. thyrsiflorus*; while roots of *R thyrsiflorus* did not show any apical ROL at a length of 4·5–5 cm, in both *R. maritimus* and *R. crispus* a considerable oxygen loss could be recorded at root lengths of 7·5–8 cm (Fig. 3b and c). With some exceptions there was a decrease in apical ROL with an increase in diffusion path length, and this was particularly evident in the *R. maritimus* profiles. The root surface oxygen concentrations were calculated (Armstrong 1979) from the ROL datasets. This revealed the enormous differences in the apical oxygen pressure between roots of comparable length of the species (Table 1), and indicates that root growth of *R. thyrsiflorus* will be restricted at a short length.

Furthermore, in *R. maritimus* the roots showed an increase in ROL from base to apex (Fig. 3c), indicating a partial loss in permeability in the wall layers of the subapical and basal root parts. This effect was clear in the shorter roots, but in longer roots and in *R. crispus* the oxygen profiles were much flatter. A reverse pattern could be observed in the roots of *R. thyrsiflorus*, where in most cases there was a decrease in ROL from base to apex (Fig. 3a). In *R. crispus* there were some exceptional oxygen profiles in short roots, beginning at the apical end of the tap-root (Fig. 3b).

Oxygen loss as related to growth and iron toxicity in plants grown on a flooded clay substrate

In the clay/sand soil used, the concentration of soluble ferrous iron in the soil solution was enhanced upon flooding to *c.* 0·2 mM in the absence of any plants (Table 2).

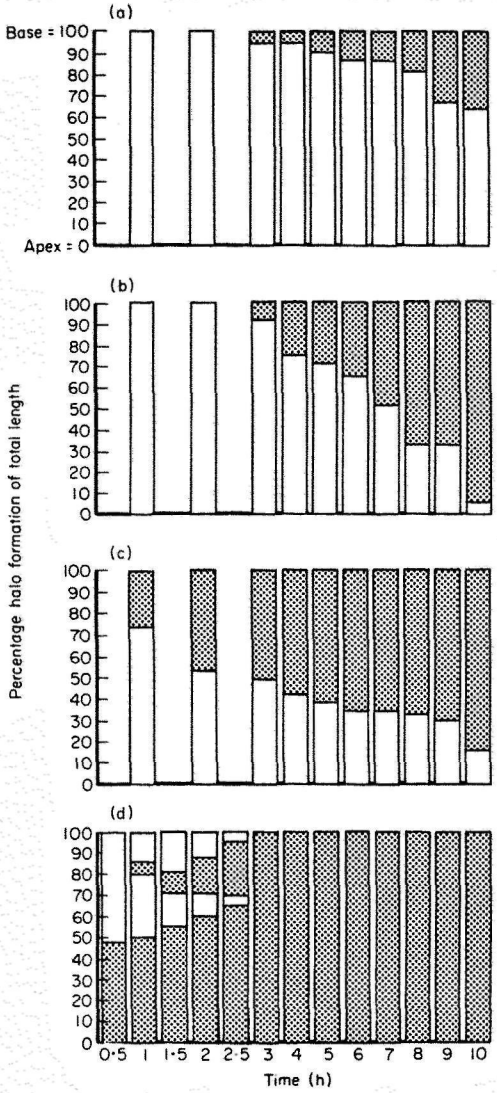

Fig. 2. Time course of halo-formation around newly formed lateral roots of *Rumex thyrsiflorus* (a), *R. crispus* (b), *R. maritimus* (c) and *Oryza sativa* (d). Data as a percentage of the total length of the root; root apex = 0%, root base = 100%; length of *Rumex* roots as in Fig. 1, length of roots ± SD of *Oryza sativa* 15·3 ± 0·7 cm.

Eight weeks after the start of the flooding treatment, the plants were harvested. The large number of new roots in *R. maritimus* had caused almost complete iron oxidation in the upper 30 cm of the soil (Table 3), clearly distinguishable because of the sharp transition zone between the oxidized (brownish) and the reduced (black) soil, and quantified by measurement of E_h and the amount of ferrous iron in the soil solution. E_h was higher in the oxidized zone (Table 3), and the amount of ferrous iron had decreased to values lower than 0·01 mM (Table 2). It should be noted that the redox potentials, whilst typical of soils showing iron oxidation, nevertheless are still indicative of mild reducing conditions and a

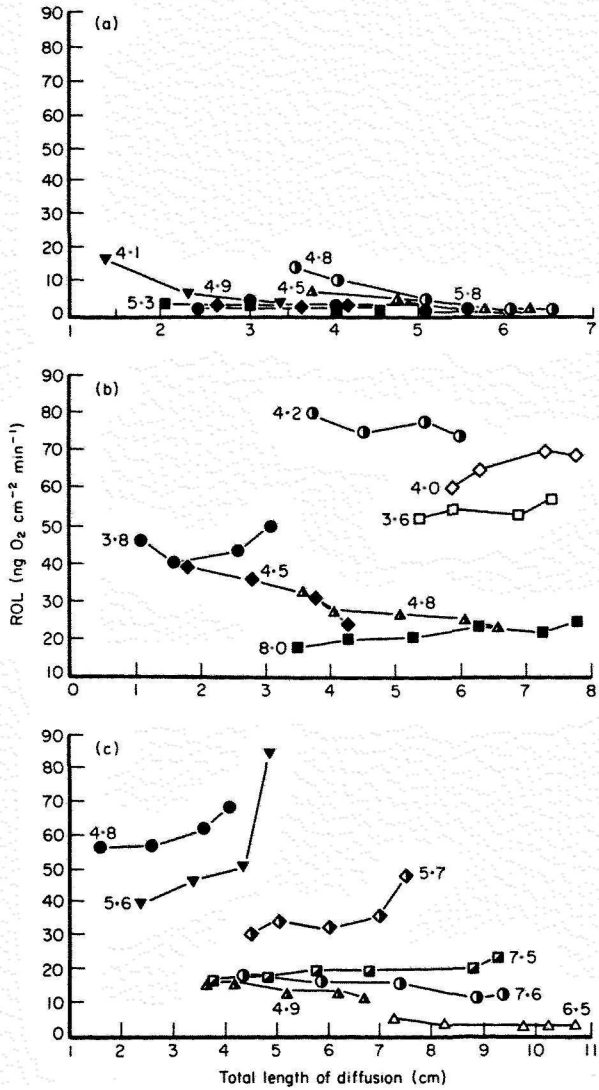

Fig. 3. Radial oxygen loss profiles of individual newly formed lateral roots of *Rumex* species with different length and originating at different positions on the tap-root as a function of the total calculated diffusion path length (a) *R. thyrsiflorus*, (b) *R. crispus*, (c) *R. maritimus*. Profiles from base (left side) to apex (right side) of individual lateral roots of several plants indicated with different symbols. The length of each lateral root is indicated at profile; mean length of tap-roots = 5 cm. The origin of the lateral roots on the tap-root: at the base (closed symbols), halfway base and apex (half open symbols) or at the apex (open symbols). Horizontal axis: total calculated diffusion path length from base of tap-root to point of measurement on lateral root.

lack of significant amounts of free oxygen. In the *R. crispus* tubes the oxidation zone was *c.* 15 cm deep, and in the tubes with *R. thyrsiflorus* only the upper 4–5 cm were oxidized (Table 3). These results illustrate the differences in soil profile oxidizing power between the *Rumex* species.

Closely connected to oxidizing power was maximal root penetration which was reached after 8 weeks. In the reduced soil layer the ROL is apparently not high enough to

Table 1. Internal oxygen pressure at 0·75 cm behind the apex of newly formed lateral roots of *Rumex* species

	Mean root length (cm)	Internal oxygen pressure (kPa)
R. thyrsiflorus ($n=5$)	5·1 ± 0·5	0·13 ± 0·04
R. crispus ($n=7$)	4·7 ± 1·5	3·2 ± 1·5
R. maritimus ($n=4$)	5·3 ± 0·5	3·7 ± 2·1

Mean of 4–7 replicates plus SD; data calculated from the ROL data as shown in Fig. 3.

Table 2. Effect of enhanced ferrous iron concentration in the soil solution on the amount of extracellular iron, precipitated on the roots and on leaf iron content of *Rumex* species, grown under drained or flooded conditions in a clay/sand soil for 8 weeks

Species/ treatment	Maximal $[Fe^{2+}]$ in soil solution (mM)	Extracellular iron precipitated on root (μg [g fresh wt]$^{-1}$)	Iron content of leaves (μmol [g dry wt]$^{-1}$)
Control (no plants)			
Drained	ND*	—	—
Flooded	0·20 ± 0·04	—	—
R. thyrsiflorus			
Drained	ND	19 ± 4	3·6 ± 0·1
Flooded			
ox.†	< 0·01	390 ± 70	4·4 ± 0·2
red.†	0·20 ± 0·04	7470 ± 1180	
R. crispus			
Drained	ND	13 ± 4	6·2 ± 0·4
Flooded			
ox.	< 0·01	80 ± 15	4·1 ± 0·1
red.	0·23 ± 0·05	4640 ± 740	
R. maritimus			
Drained	ND	16 ± 4	5·7 ± 0·2
Flooded			
ox.	< 0·01	45 ± 14	19·9 ± 0·4
red.	0·21 ± 0·05	4660 ± 650	

Means of five replicates ± SD.
*ND, not determined; †ox. = oxidized, red. = reduced soil layer.

oxidize the soil, and some deeper root growth stops completely, after which the tips turn black indicating death. The differences between the species result from the differences in the development of an aerenchyma system, the balance between internal diffusive resistance, distribution and the degree of respiration and oxygen leakage (Laan *et al.* 1989; P. Laan unpublished results). As a consequence, the apical oxygen concentration differs significantly for any given length of root (Table 1), and hence root penetration of

Table 3. Differential effect of internal aeration and concomitant oxidizing power of newly formed laterals of *Rumex* species on root penetration and the redox state of a clay/sand soil after 8 weeks of flooding

Species	Depth of oxidized soil layer (cm)	Maximal root depth (cm)	E_h (mV)	
			Oxidized soil layer	Reduced soil layer
Control (no plants)	0·0	—	—	−203±2
R. thyrsiflorus	4·6±0·5	11·8±2·5	55±5	−183±6
R. crispus	15·4±1·8	29·0±1·6	173±6	−183±15
R. maritimus	29·6±2·5	36·6±1·7	183±15	−163±6

Means of five replicates ± SD.

R. maritimus was almost maximal (Table 3), while in *R. thyrsiflorus* root penetration was poor and only a few roots could be found at a depth of 10–12 cm (Table 3).

Just below or above the transition zone between the oxidized and the reduced soil, iron plaque formation could be observed on the roots (Fig. 4). In *R. thyrsiflorus*, where there is only poor oxygen loss, the rhizoplane was oxidized predominantly. The iron plaque contained 7470 μg iron per gram fresh roots, a higher value per unit fresh weight of roots than in *R. crispus* and *R. maritimus* (Table 2). It should be emphasized, however, that the diameter of the *R. crispus* and *R. maritimus* roots in most cases was twice as high as those of *R. thyrsiflorus*. Therefore, these figures do not indicate a greater degree of plaque per unit length between the species, but serve to illustrate the ability of even *R. thyrsiflorus* to bring about significant iron oxidation. In *R. maritimus* and to a lesser extent also in *R. crispus*, which both showed high radial oxygen losses, in addition to plaque formation, an obvious remote oxidation around individual roots was readily observed in the oxidized zone close to the transition zone. Just above the iron plaques and sometimes overlapping the plaque, micropedotubules were formed (Brewer 1964): cylinders of iron precipitations plus sand particles at a few millimetres distance from the root surface, leaving the root itself fairly clear from iron precipitates. It is this remote oxidation which must ultimately have led to the generalized oxidation of the profiles, and since the upper 4–5 cm of *R. thyrsiflorus* sediment was visibly oxidized throughout, this also indicates a long-term remote oxidation, although signs of this could not be discovered around individual roots.

Roots from the oxidized soil layer, which did not show visible plaque formation, were also analysed. In all investigated species an increase in iron content of the flooded versus the drained plants was recorded (Table 2), probably due to precipitation in extracellular (apoplastic) space (Armstrong & Boatman 1967; Green & Etherington 1977). This increase was highest in *R. thyrsiflorus* and much lower in both *R. crispus* and *R. maritimus*. It is interesting to note that the differential behaviour of the root systems, as stated above, did not result in differences of iron content of the leaves on the basis of dry weight in both *R. thyrsiflorus* and *R. crispus*, which appeared to be as low as in the drained controls (Table 2). In *R. maritimus*, however, a fourfold increase was recorded.

Presumably as a consequence of the differences in root growth and concomitant soil oxidation, the biomass production and nutrient content of the shoots was variably

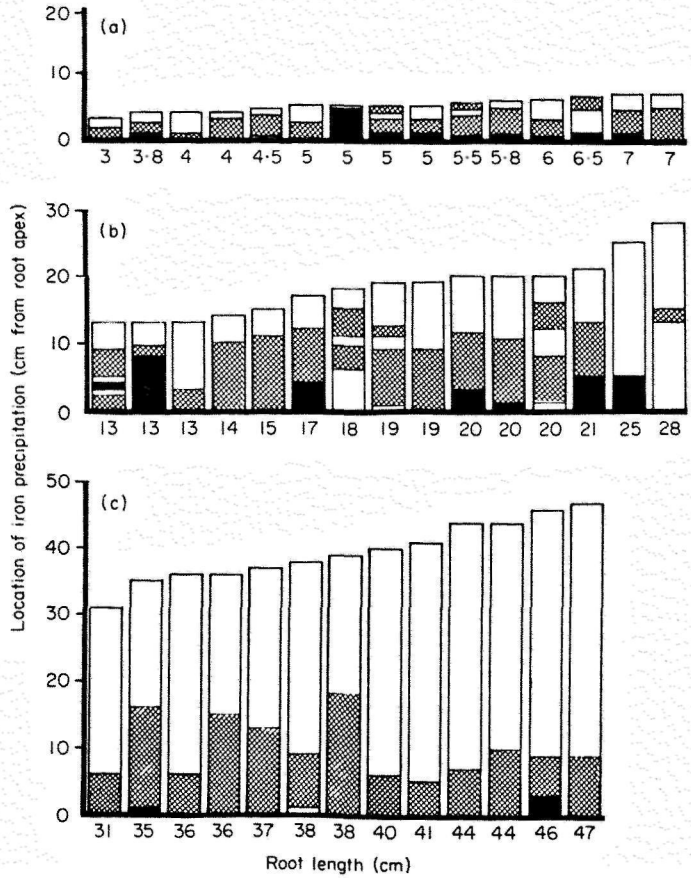

Fig. 4. Location of iron precipitation on newly formed roots of *Rumex thyrsiflorus* (a), *R. crispus* (b) and *R. maritimus* (c) with different length. (□) No iron precipitation, (▩) iron oxide plaque formed, (■) ferrous sulphide blackening. Plants grown on a flooded clay for 8 weeks in tubes with a length of 40 cm.

affected (Table 4). In *R. thyrsiflorus* and *R. crispus*, there was a general decrease in nutrient content upon flooding. However, shoot biomass production of *R. thyrsiflorus* was halved upon flooding, whilst the biomass production of *R. crispus* was hardly affected (Table 4). Thus, the lower nutrient content of *R. crispus* shoots did not result in a decrease of biomass production, as it did in *R. thyrsiflorus*. In *R. maritimus*, shoot biomass production was very high and not affected by flooding; also the nutrient content of the leaves, although already low in the drained plants, was not affected, except for potassium, which was halved.

DISCUSSION

The results show that roots of the three investigated *Rumex* species differ in oxidative power. All the species were able to oxidize methylene blue in solution and iron in flooded soil, but the roots developed in the anaerobic conditions were better able to effect these

Table 4. Effect of flooding on biomass production and on the nutrient content of leaves of *Rumex* species

Species/ treatment	Shoot dry weight (g)	Leaf nutrient content (μmol [g dry wt]$^{-1}$)				
		Nitrogen	Phosphorus	Potassium	Calcium	Magnesium
R. thyrsiflorus						
Drained	15·2±1·4	1878±45	143±10	1238±37	323±11	435±22
Flooded	7·2±1·4	1006±64	44±2	355±15	190±2	218±3
R. crispus						
Drained	13·6±1·4	1372±54	90±5	857±39	532±27	311±7
Flooded	12·7±2·5	702±35	58±10	347±23	315±13	160±8
R. maritimus						
Drained	24·8±2·6	1018±91	59±2	478±11	615±19	401±5
Flooded	25·4±3·9	1052±52	59±2	272±10	761±40	398±11

Means of five replicates plus SD; plants grown in a clay/sand mixture for 8 weeks.

oxidations. The results with methylene blue (Fig. 2) indicate that there is significant permeability to oxygen along the whole length of the *Rumex* roots. This contrasts with patterns detected in rice here and elsewhere (Armstrong 1971).

As in the methylene blue experiments, the ROL profiles showed differences in oxygen losses between the species (Fig. 3); they are consistent with the differences in porosity, aerenchyma formation and respiratory demand along the roots (Laan *et al.* 1989; P. Laan unpublished results). Oxygen losses at the apices were high in *R. maritimus* and *R. crispus* and low in *R. thyrsiflorus*. It must be emphasized, however, that the rate of the ROL at the apices is very dependent upon the length of the root and its position on the tap-root. In general, the ROL decreased with increasing length, but the ROL patterns along the roots varied considerably among the species. In *R. maritimus*, the ROL tended to increase towards the apex, in *R. crispus* it was constant over the whole length of the root. In *R. thyrsiflorus* oxygen loss tended to decrease towards the apex. Apparently, in *R. maritimus* there was an obvious basipetal decrease in root wall permeability in the aging roots, in *R. crispus* this was less pronounced; in *R. thyrsiflorus* a decrease in root wall permeability could only be deduced by calculation.

The best correlation between the ROL and root length was obtained when root length was taken as the total diffusion path length between the base of the tap-root and the place of measurement (Fig. 3). Thus, in *R. maritimus* the basally attached roots showed high oxygen losses, and the more apical ones had much lower values. In *R. crispus* some exceptions were observed: some short laterals, originated at the apical ends of the tap-root, showed higher oxygen losses. This suggests that the tap-root of *R. crispus* did not show much diffusive resistance.

The differences described above explain, at least partly, the differential capacity of the root systems to oxidize the soil. Apical oxygen concentration is causally correlated to root growth (Armstrong 1979; Armstrong & Webb 1985), and differences in aerenchyma formation and subsequent internal aeration should result in a differential root growth and development. As oxygen loss was correlated to aerenchyma formation, the major effect observed is a differential soil iron oxidation depth.

With respect to iron toxicity, *R. thyrsiflorus* does not oxidize deeper soil layers, but the long-term oxidation of the shallower depth is apparently sufficient to detoxify that region in terms of iron. On the other hand, in *R. maritimus* shoot iron levels rose fourfold upon flooding, while biomass production was unaffected. Leaves of rice that clearly showed iron toxicity symptoms contained 8–20 μmol (g dry wt)$^{-1}$ (Yoshida & Tadano 1978; Ottow *et al.* 1982); *R. maritimus*, with healthy growing shoots and with leaves, containing 20 μmol (g dry wt)$^{-1}$, is apparently less sensitive to high iron levels in the leaves under these conditions. It is therefore unlikely that iron toxicity is a determinant of flood-tolerance in the investigated *Rumex* species; even if the soil solution iron levels had been higher, iron toxicity might have been more likely to occur in the flood-tolerant *R. maritimus* than in the relatively flood-intolerant *R. thyrsiflorus*.

It may seem surprising to find that the highest leaf iron content was recorded in the most flood-resistant *R. maritimus*, i.e. a high oxygen loss does not automatically lead to a low iron uptake. It should be taken into consideration, however, that iron uptake and iron exclusion are probably closely related to the growth rate of the roots. The concentration of ferrous at the site of uptake at the root is the result of, on the one hand, the flux of ferrous to those sites, and on the other of the oxidation rate by micro-organisms and the root itself (Bienfait 1989). The high growth rate of *R. maritimus* will only be maintained when water uptake, needed to counteract transpiratory loss, is high, and the flux of ferrous to the roots is therefore high. The half-life of ferrous in the presence of atmospheric oxygen at pH 7 is in the order of 30–60 min (Davison & Seed 1983). Moreover, at a fast growing root tip, microbial growth and activity are not yet fully developed, so that a bacterial contribution to ferrous oxidation is low. Thus, the net oxidation rate of ferrous at and just behind the tips of fast growing roots may be well below the ferrous supply rate, which then leads to high iron concentrations in the shoots.

In *R. thyrsiflorus*, the opposite situation is found: root growth is slow, soil oxidation is poor, but the ROL is sufficient to form iron plaques over the whole root surface (Fig. 4), where growth and activity of microbes have had time to establish, so that they can increase the local ferrous oxidation capacity. Thus, iron toxicity is not a problem. But in this case it is likely that soil exploitation is restricted and nutrient uptake strongly diminished. Moreover, the iron plaque formed on the root surface (Fig. 4) can bind considerable amounts of nutrients (Otte *et al.* 1989). A general nutrient decrease in the leaves was indeed recorded (Table 4), and a decrease in potassium, calcium and phosphorus content was obvious.

Finally, in *R. crispus* a general decrease in nutrient content was also observed, but this did not result in a decrease in biomass production. Apparently, soil exploitation was not significantly restricted and nutrient levels were not low enough to decrease the yield. Since the leaf nutrient content in the drained treatments differed significantly between the species, it may be assumed that nutrient deficiency levels in *R. thyrsiflorus* are reached at higher concentrations than in *R. crispus* and *R. maritimus*.

We propose that the relative flood-intolerance of *R. thyrsiflorus* is predominantly caused by a lack of root growth and concomitant nutrient deficiency. Experiments with higher soluble iron concentrations will determine whether the growth rate of *R. maritimus* may be affected ultimately by the soil solution iron concentration.

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CHAPTER 4 Internal oxygen transport in *Rumex* species and its significance for respiration under hypoxic conditions

with M. Tosserams, C.W.P.M. Blom & B.W. Veen
Plant and Soil (1990) **122**: 39-46 (with permission)

CHAPTER 5 Oxygen uptake by roots of *Rumex* species at different temperatures: the relative importance of diffusive resistance and enzyme kinetics

with M. Tosserams, P. Huys & H.F. Bienfait

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Internal oxygen transport in *Rumex* species and its significance for respiration under hypoxic conditions

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Abstract

Rumex thyrsiflorus, *Rumex crispus* and *Rumex maritimus* show a differential flood-tolerance in the river ecosystem in the Netherlands. *R. thyrsiflorus* occurs at high-elevated habitats and is flood-intolerant, the other two species occur at lower-elevated habitats and are flood-tolerant. We compared their respiratory activity under aerobic and anaerobic conditions in the root environment and quantified the internal gas transport. The results indicate that aerial oxygen can be used for root respiration in both aerobically and anaerobically grown plants. The amount of oxygen used via internal aeration increased with decreasing oxygen concentration in the root environment. Aerobically grown plants of *R. maritimus* and *R. crispus* already showed a high internal aeration, but there was a significant increase in internal oxygen transport in anaerobic plants, where new, aerenchymatous roots had formed. This indicates the functional significance of new root formation for respiration in these species upon hypoxia. After two weeks of anaerobiosis, more than 50% of the total respiration of the roots of young plants of *R. maritumus* and 40% of roots of young plants of *R. crispus* was due to internal aeration at low oxygen concentrations in the root environment.

In *R. maritimus* both young and old plants performed in this way, in *R. crispus* only young plants, while *R. thyrsiflorus* showed some internal aeration, but this was hardly detectable. These differences can be explained on the basis of a different morphology and concomitant diffusive resistance of both root and shoot system.

In experiments with different submergence levels of the shoot, the amount of internal aeration was positively correlated to the total leaf area protruding above the water surface in *R. maritimus*. This indicates a functional significance of the petiole and leaf elongation response upon total submergence of this species.

Introduction

Upon exposure to anaerobic conditions, many plant species form aerenchyma in their roots (Arber, 1920; Armstrong, 1979; Jackson and Drew, 1984; Justin and Armstrong, 1987; Konings and Verschuren, 1980; Laan *et al.*, 1989a). This response is supposed to be beneficial to plant growth or survival under situations of root inundation. Aerenchyma development increases root porosity, hence reduces the resistance to diffusive oxygen transport from shoot to root (Armstrong, 1979; Veen, 1989), so that aerobic respiration can be maintained in roots when the root environment becomes anoxic (Armstrong, 1979; Armstrong and Gaynard, 1976; Drew *et al.*, 1985; Lambers *et al.*, 1978; Prioul and Guyot, 1985).

Wheat plants can adapt to root anaerobiosis within a week by aerenchyma formation (Prioul and Guyot, 1985; Wiedenroth and Erdmann, 1989). Prioul and Guyot quantified the internal oxygen transport from the shoot to the root system

of anaerobically grown wheat plants. However, to allow internal aeration, the formation of an extended aerenchyma system is not strictly necessary. Armstrong *et al.* (1982; 1983) showed that pea roots with a porosity of only 2—4% showed internal aeration, and Armstrong (1979) and De Willigen and Van Noordwijk (1984) calculated that even effective porosities of about 1—4% can contribute significantly to root respiration under hypoxia in the root environment, provided that the gas-filled pores form a continuous system. Consequently, root respiration, measured as oxygen depletion from the root environment will be underestimated when additional oxygen is internally transported from the shoot to the root.

The aim of this study was to investigate the importance of internal gas transport for root respiration in three *Rumex* species, which occur in the river ecosystem in the Netherlands and show a differential response towards flooding (Laan *et al.*, 1989a;b). Their root system consists of a tap-root, from which laterals are formed. Upon flooding new laterals develop; the tolerant species *R. maritimus* and *R. crispus* both develop an aerenchyma system in the new lateral roots (Laan *et al.*, 1989a).

In the river ecosystem both *R. maritimus* and *R. crispus* are infrequently confronted with total submergence during the growth period of the plants (Van de Steeg, 1984; Voesenek *et al.*, 1989), a situation in which no efficient use can be made of the aerenchyma system. As a response both laminae and petioles elongate so that the water surface is reached (Voesenek and Blom, 1989). We investigated whether the functional significance of this elongation may be to serve as a base for the internal aeration of the root systems.

Materials and methods

Plant growth

Seeds of *Rumex thyrsiflorus* Fingerh., *R. crispus* L. and *R. maritimus* L. were collected from natural populations and sown on black polyethylene granules (stamylan LD, DSM, The Netherlands). After germination, which occurred within one week, the plants were separated into two batches.

One batch, used for the experiments with young plants, were allowed to grow on aerated hydroculture for 7 weeks (aerobically grown plants), with the following nutrient composition: Macronutrients: KNO_3 1 m*M*, $Ca(NO_3)_2$ 1 m*M*, $NaNO_3$ 1 m*M*, KH_2PO_4 0.5 m*M*, $MgSO_4$ 0.25 m*M*; Micronutrients: FeEDTA 0.25 m*M*, KCl 12.5 μM, H_3BO_3 6.3 μM, $MnSO_4 \cdot H_2O$ 0.5 μM, $ZnSO_4 \cdot 7H_2O$ 0.5 μM, $CuSO_4 \cdot 5H_2O$ 0.1 μM, H_2MoO_4 0.1 μM in a growth room (22°C; 16 h light at 200 μmol m^{-2} s^{-1} PAR, 8 h dark; R.H. 60%). Some of these plants were transferred to a stagnant, anaerobic (0.1% agar (w:v)) nutrient solution after 5 weeks and kept there for one to two weeks. In this period new laterals developed from the tap-root (anaerobically grown plants). All nutrient solutions were changed every two days and maintained at a constant pH of 5.5

Another batch (old plants) was allowed to grow for eight to ten weeks in a growth room (temperature 24°C; light intensity 500 μmol $\cdot$ $m^{-2} \cdot s^{-1}$ PAR for 16 h, 8 h dark; R.H. 70%); the nutrient solution was changed twice a week. Some of these plants were transferred to an anaerobic 0.1% agar in $\frac{1}{2}$ × full strength nutrient solution and both aerobic and anaerobic plants were allowed to grow for another one to two weeks in a growth chamber at the same light and temperature conditions. In this period the anaerobic plants developed a new lateral root system.

Experimental assembly

The experimental setup, used for the old plants, was derived from a measuring system used by Veen (1977) (Fig. 1a). A plant was placed in the system, with its roots in the root vessel (*RV*). Closed-cell rubber foam sealed the opening between the plant and the stopper at the upper side of the root vessel. Over the shoot of the plant a 'Perspex' cover (*K*) was mounted, through which air or nitrogen gas was blown. With a small rotary pump (*P*) (Eheim type 1018) a thermostatted (*C*) nutrient solution with a total volume of 3 liters was circulated at 1 L $\cdot$ min^{-1}. In the measuring chamber (*M*) a galvanic lead/silver electrode (Precision Scientific Inc.) was used to register the oxygen concentration. When the magnetic valve S_1 was opened and S_2 closed, the flow of water fell over a distance of about 10 cm into a vessel (*A*), which gave an adequate aeration of the nutrient solution. The

oxygen concentration at the start of each experiment varied depending on root size. When S_2 was opened and S_1 closed, the oxygen supply to the nutrient solution was prevented and uptake rate of oxygen by the plant roots was recorded at 5-min intervals using a HP 85 computer with a 3421A-data acquisition unit. From the depletion curve the relation between oxygen concentration and uptake was calculated.

The measuring system, used for the young plants (Fig. 1b), was comparable to the one used for the old plants. Instead of 3 liters, the net volume of the root vessel was 180 mL; a polarographic oxygen electrode according to Kimmich and Kreutzer (1969) was placed into the root vessel (*RV*) and optimal mixing of the nutrient solution was achieved by stirring with motor-driven paddle-boards (*ST*), together with the positioning of three vertical baffles at the inner side of the root vessel (*IB*). The shoot was separated from the root system by two crescent 'Perspex' lids, sealed with clay and lanolin. The shoot was covered with a 'Perspex' lid (*K*) and darkened by a black plastic cover (*B*); this 'Perspex' cover allowed the flow-through of air or nitrogen gas, or of water used to submerge the shoot to different levels. A thermostatic waterbath (*W*) (Hakke, type G-D8) contained 1 litre of the aforementioned nutrient solution (*NS*) plus 1% (w/v) glucose, which was aerated with a porous aeration-stone (*G*) and maintained at 25.0°C. After aeration for *ca.* 30 min, this nutrient solution was pumped into the inner root vessel (*RV*) with a peristaltic pump, and the vessel was closed. Oxygen consumption of the root system was calculated from a depletion curve, measured with an oxygen micro-sensor (Diamond Electro-Tech, Ann Arbor, Mich., USA). The nutrient solution was renewed after each measurement (*ca.* 50 min) to prevent a significant bacterial contribution to respiration. The first depletion curve was always made with air circulated through the shoot compartment, the second with a nitrogen gas flow. Changes from air to nitrogen gas were completed within one minute; depletion experiments could therefore be performed within 2 — 3 minutes. At the end of each set of measurements, the shoot was cut off and the remaining tap-root sealed with a mixture of clay and lanolin, after which the depletion of oxygen was measured.

The absolute zero oxygen point was checked

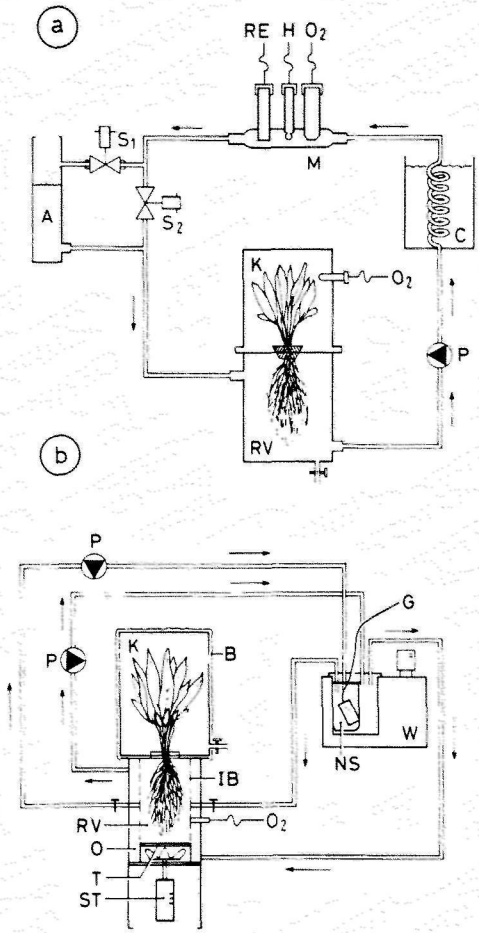

Fig. 1. Diagram of the measuring system for old plants **(a)** and for young plants **(b)**. RV = root vessel, K = perspex cover, P = pump, C = cooling bath, S = solenoid valve, A = aeration vessel, M = measuring chamber consisting of a reference electrode (RE), a pH electrode (H) and an oxygen electrode (O_2); B = black plastic cover, W = thermostatic waterbath, from which two flow-through systems are circulated: one via the inner root vessel (RV) and the other (cooling system) via the outer compartment (O), NS = nutrient solution, aerated by gas inlet (G), T = perforated table, ST = stirrer, IB = inner baffles to optimize mixing of the nutrient solution.

both by Winkler-titration and by measurement of nutrient solution, which was thoroughly bubbled with nitrogen gas.

To prevent the entry of photosynthetic oxygen, all experiments were performed in the dark. When starting the experiments immediately after the change from the light to the dark period, the first

depletion curve always showed a higher uptake rate of oxygen from the nutrient solution than the following depletion curves of the same plant, at least at the higher concentrations. Further experiments therefore were started after at least one hour in the dark.

After each set of experiments new lateral roots were separated from old ones and from tap-roots; dry weight of these root parts was determined after drying (48 h, 70°C). In the submergence experiments, leaves and petioles were severed at the different submergence levels and leaf area was measured with an area meter (MOP Kontron GMBH).

Determination of pore-space resistance

Pore-space resistance of 7-cm petiole segments of anaerobically grown plants was determined by measuring the time needed to move 4 cm^3 of air through the petioles, using a burette filled with water, giving a pressure difference of 5.5 kPa. From the flow-rate the pore-space resistance was calculated according to Armstrong (1979) and Armstrong *et al.* (1988).

Results

Oxygen uptake by Rumex root systems was independent of the oxygen concentration in the surrounding medium down to *ca.* 50 μM (Critical Oxygen Pressure for Respiration in the measuring system (= COPR), Fig. 2). Higher oxygen depletion rates were obtained with nitrogen gas than with air in the shoot compartment; oxygen uptake rates with nitrogen gas in the shoot compartment and those of decapitated plants were similar and represent the actual respiration rate of the root systems. Respiration depending on internal oxygen transport was calculated as the difference between oxygen depletion rates of roots of intact plants supplied with air in the shoot environment and that of decapitated plants, down to the COPR.

With young, aerobically grown plants of both *R. maritimus* and *R. crispus* (Fig. 2a,c), the oxygen

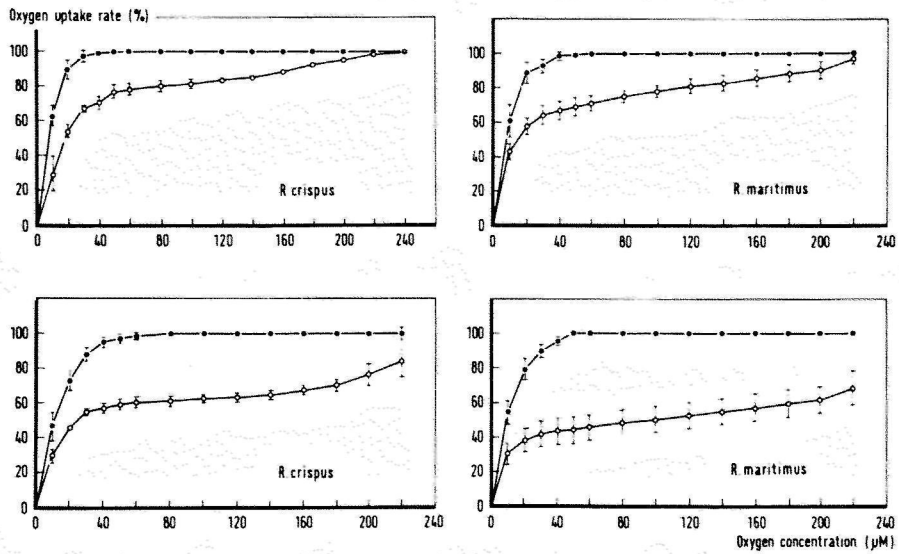

Fig. 2. Effect of air in the shoot compartment (○) or removal of the shoot (●) on the oxygen consumption from the root environment by root systems of *Rumex maritimus* and *R. crispus* plants, grown aerobically (*top*) or after 1—2 weeks anaerobiosis (*bottom*). (Data as a percentage of maximal respiration rate at 220 μM O_2 = 100%, being 192 ± 8 (aerobic *R. maritimus*), 192 ± 44 (anaerobic *R. maritimus*), 175 ± 54 (aerobic *R. crispus*) and 167 ± 13 (anaerobic *R. crispus*) µmol O_2 $(g\,dry\,wt)^{-1}\,hr^{-1}$; means of 3—4 replicates ± SE; measurements performed after at least one hour in the dark; age of plants 5 weeks plus 1—2 weeks anaerobic treatment.

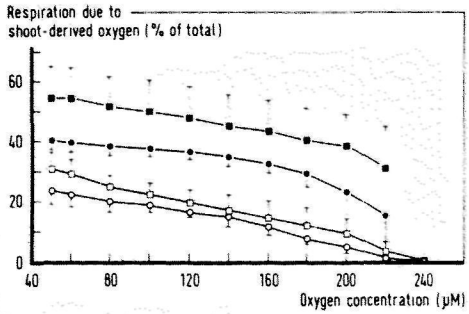

Fig. 3. Contribution of shoot-derived oxygen to root respiration (as % of total respiration) at different oxygen concentrations in the root environment of aerobically (*open symbols*) and anaerobically (*closed symbols*) grown *R. maritimus* (□, ■) and *R. crispus* (○, ●) plants (means of 2 (aerobic) or 4 (anaerobic) plants ± SE; age of the plants 5 weeks (young, aerobic plants) plus 1—2 weeks anaerobiosis (young, anaerobic plants))

uptake rate of plants with air in the shoot compartment decreased with decreasing oxygen concentration around the roots (Fig. 2a,c), indicating that aerial oxygen was increasingly used for root respiration. The depletion curves of young anaerobically grown plants (Fig. 2b,d), which had developed a new aerenchymatous lateral root system, showed similar patterns as those obtained from the aerobic plants. Here, internal oxygen transport could be observed in both *R. maritimus* and *R. crispus*, already at high solution oxygen concentrations, and again increasing with decreasing oxygen concentration.

Figure 3 shows the contribution of shoot-derived oxygen to root respiration in young aerobically grown plants and in anaerobically grown plants, with different amounts of newly formed, aerenchymatous roots. Two phenomena were observed for both *R. maritimus* and *R. crispus*: 1. Internal oxygen transport occurs in both aerobic and anaerobic plants and increase with decreasing oxygen concentrations in the root environment, and 2. The longer the period of anaerobiosis (and thus the more new laterals had developed), the higher the amount of internal oxygen transport (data incorporated in mean values, shown in Fig. 3). At low solution oxygen concentrations, more than 50% of the total respiration was supplied by shoot-derived oxygen in anaerobically *R. maritimus* plants, which had developed a large number of new laterals, in *R. crispus* this was at least 40% (Fig. 3, Table 1).

In *R. thyrsiflorus* internal aeration was very low, and no differences could be observed between aerobically and anaerobically grown plants (data not shown).

The responses of older plants differed from those of the young ones, in that older aerobically grown *R. maritimus* and *R. crispus* plants did not show internal oxygen transport (Table 1). With anaerobically grown old plants, internal aeration could only be observed in *R. maritimus*, and became apparent only at lower solution oxygen concentrations ($< 100\,\mu M$ O_2, data not shown). At the COPR (*ca.* $55\,\mu M$ O_2), at least 71% of the total respiration was due to shoot-derived oxygen (Table 1). In older, anaerobic *R. crispus* plants, there were no clearcut differences between the depletion curves

Table 1. Contribution of shoot-derived oxygen to root respiration at the COPR value (*ca.* $55\,\mu M$ O_2) of young and old aerobically and anaerobically grown *Rumex maritimus* and *R. crispus* plants. Data as percentages of respiration rates at $200\,\mu M$ O_2 (= 100%), being: *R. maritimus*: young aerobic 192 ± 8, old aerobic 68 ± 6, young anaerobic 192 ± 44, old anaerobic 75 ± 10, *R. crispus*: young aerobic 175 ± 54, old aerobic 70 ± 8, young anaerobic 167 ± 13, old anaerobic 56 ± 4 μmol oxygen (g dry wt)$^{-1}$ hr^{-1}; means of 2 (aerobic plants) or 4 (anaerobic plants) replicates ± SE; age of young plants 5 weeks (aerobic plants), or 5 weeks plus 1—2 weeks anaerobiosis (anaerobic plants); old plants 13 weeks (aerobic plants), or 13 weeks plus 1 week anaerobiosis (anaerobic plants)

Species/age	Contribution of shoot-derived oxygen to respiration (%)	
	Aerobically grown plants	Anaerobically grown plants
R. maritimus		
young	29 ± 5	54 ± 8
old	0	71 ± 6
R. crispus		
young	22 ± 4	39 ± 2
old	0	7 ± 3

of plants with air in the shoot compartment and of decapitated plants, hence internal aeration is of minor importance (7%, Table 1).

To evaluate the ecological significance of the internal aeration process, the depletion of oxygen was measured with anaerobic *R. maritimus* plants, that were submerged to different degrees (Fig. 4). The extent of internal oxygen diffusion was determined by calculating the differences between oxygen uptake rates at the different submergence levels and of decapitated plants at the COPR-value (Fig. 5). Plants that were totally submerged showed characteristics of oxygen depletion from the root environment that were similar to those of decapitated plants. With more leaf area protruding above the water surface, the importance of internal oxygen transport increased (Fig. 5). Submergence of leaf bases and petioles resulted in a relatively sharp decrease of the apparent respiration due to internal aeration from 61 to 38% (Fig. 5), suggesting a high permeability to oxygen of these shoot parts.

Discussion

Aerial oxygen can be used for root respiration (Figs. 2, 3) and this process can contribute significantly in maintaining aerobic respiration under situations of root inundation and of partial

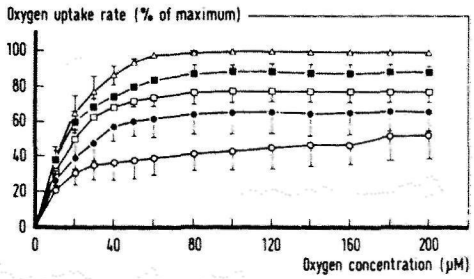

Fig. 4. Oxygen depletion profiles of anaerobically grown *R. maritimus* plants, with new lateral root system developed, at different submergence levels of the shoot (in percent of maximum oxygen uptake rate of decapitated plants at 200 μM O_2 = 100%, being 263 ± 28 μmol O_2 (g dry wt^{-1} hr^{-1}) (△ = water level 28 cm (totally submerged) and decapitated plants, ■ water level 20 cm, □ = water level 13 cm, ● = water level 7 cm, ○ = water level 0 cm (air) above shoot base (age of the plants 6 weeks plus 10 days anaerobiosis; means of 2 replicates ± SE; plants measured with shoot in the dark).

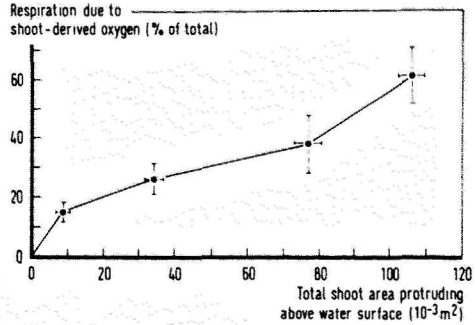

Fig. 5. Relation between root respiration due to internal oxygen transport and total area of the shoot protruding above the water surface of anaerobically grown *R. maritimus* plants at the COPR-value in the measuring system = *ca.* 50 μM oxygen; means of 2 replicates ± SE; total area of shoot material 106.1 ± 2.6 × 10^{-3} m^2).

submergence (Figs. 4, 5). The percental contribution of shoot-derived oxygen to respiration varied from at least 40% in young anaerobically grown *R. crispus* to at least 71% in old anaerobically grown *R. maritimus* plants (Fig. 3, Table 1).

The amount of internal oxygen supplied to the roots becomes more important with decreasing oxygen concentration in the root environment (Fig. 3). Although the oxygen uptake at COPR of roots of plants with air in the shoot environment is reduced compared to decapitated plants, the actual root respiration can be considered maximal and equal to the oxygen requirement of the roots of decapitated plants. Therefore, a combination of internal aeration and oxygenation from the root medium can completely satisfy the needs of the root system for oxygen, at least down to the COPR (= 50—60 μM). Unfortunately, the system does not allow quantification of internal aeration below the COP-value, since we do not know the actual respiration rate of the root system below COPR. However, because oxygen gradients within the plant increase with decreasing oxygen concentration in the outer solution, it is justified to state that internal aeration below COP will at least be the same and probably higher than at solution oxygen concentrations above the COP-value. Thus, internal aeration increases to 100% when oxygen is completely depleted from the root medium, regardless of the actual respiration rate.

From these results, the functional significance of the elongation of leaves and petioles of *R.*

maritimus upon total submergence, becomes apparent: by reaching the water surface, aerobic respiration can be restored through internal oxygen transport (Figs. 4, 5). Comparable results were found by Gaynard and Armstrong (1987) with *Eriophorum angustifolium* and by Atwell *et al.* (1982) with rice seedlings.

A period of anaerobiosis increased internal aeration in *R. crispus* and *R. maritimus*, when oxygen supply from the root medium is restricted (Fig. 3). The most striking response to anoxia is the formation of a new, aerenchymatous root system (Laan *et al.*, 1989a). Thus, the importance of internal aeration seems to be closely related to the formation of a new, aerenchymatous lateral root system. This was especially true for *R. maritimus*, where both young and old plants performed in this way (Table 1). Upon ageing, the leaves, tap-root and lateral roots retain a continuous porous system in this species. For *R. crispus*, however, the situation is more complicated; whilst comparable responses as in *R. maritimus* were found in young plants, older plants showed virtually no internal aeration (Table 1). In *R. crispus*, the amount of new laterals formed, their growth rate, and thus their sink activity, decreases with age, and the influence of the woody tap-root becomes increasingly important as sink for growth.

Differences in the capability of internal oxygen transport can be explained in terms of internal diffusive resistance (Armstrong, 1979; Gaynard and Armstrong, 1987), and in case of the *Rumex* species this holds for the tap-root, lateral roots and petioles. Since formation of aerenchyma reduces diffusive resistance in lateral roots (Armstrong, 1979), and aerenchyma formation takes place to the same extent in *R. maritimus* and *R. crispus* (Laan *et al.*, 1989a), the different response of the species (Table 1) must be caused by morphological differences elsewhere than in the lateral roots. Differences in tap-root porosity most likely explain the fact that old plants of *R. maritimus* are well-aerated internally, while those of *R. crispus* are not: both young and old tap-roots of *R. maritimus* have an 'open' structure, are very spongy, and as a consequence porosity is high. The tap-root of old *R. crispus* plants is much more woody and porosity is low. In young *R. crispus* plants, many new lateral roots are formed, but with age, the resistance of the woody tap-root becomes increasingly important and fewer new, aerenchymatous roots develop. Although the tap-roots of young *R. crispus* plants are much less porous than those of *R. maritimus*, they are apparently open enough to enable internal gas transport (Fig. 2; Laan *et al.*, 1989b). With age, the capability of internal gas transport is lost, because the tap-root with its high diffusive resistance, is increasingly inhibiting this process. These observations fit in the idea that *R. crispus* plants, upon ageing, rely more on dormancy for survival than *R. maritimus*, which, at all ages, seems to be completely dependent on oxygen availability.

Table 2. Effective pore-space resistance (in $s cm^{-3} \times 10^5$) of 7-cm petiole segments of anaerobic *Rumex* species, illustrating the differences in continuity of gas-filled pores (means of 3 replicates ± SE)

Rumex maritimus	1.0 ± 0.3
Rumex crispus	1.5 ± 0.5
Rumex thyrsiflorus	19.7 ± 1.8

In the flood-intolerant *R. thyrsiflorus* there is no morphological basis for extensive internal gas transport: it possesses a woody tap-root with low porosity and does not form aerenchyma in the lateral root system (Laan *et al.*, 1989a). In addition, petioles of this species lack a continuity of gas-filled pores (Table 2).

The phenomenon that young aerobically grown plants of *R. maritimus* and *R. crispus* perform internal aeration equally well (Fig. 2) can be explained by assuming relatively low diffusive resistances throughout the plant, plus a high respiratory sink-activity of the lateral roots: in young plants, respiratory demand is much higher than in old plants (Table 1; Laan and Lambers, unpubl. data; Van der Werf *et al.*, 1988). Together with the fact that tap-roots are more porous, hence diffusive resistance is lower, these features together must have permitted considerable internal gas transport. On the other hand, with low diffusive resistances and a lowered sink activity, as was the case in old *R. maritimus* plants, a reverse gas transport (from root to shoot) is likely to occur when the shoot cover contains nitrogen gas. Indeed, this was recorded in old, anaerobically grown *R. maritimus* plants at solution oxygen concentrations higher than 100 μM.

In conclusion, the results show that internal longitudinal oxygen transport can be of considerable importance in maintaining aerobiosis in the

root system under hypoxic conditions; as a consequence this phenomenon can be a crucial factor in the flood-tolerance of the *Rumex* species.

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OXYGEN UPTAKE BY ROOTS OF *RUMEX* SPECIES AT DIFFERENT TEMPERATURES: THE RELATIVE IMPORTANCE OF DIFFUSIVE RESISTANCE AND ENZYME KINETICS

SUMMARY

Oxygen uptake characteristics of the roots of three *Rumex* species were compared, and related to kinetics of the respiratory system and to root anatomy. The observed differences could not be explained from differences in basal characteristics of the oxygen uptake system: in all three species, cytochrome-mediated respiration contributed 70% and cyanide-insensitive (alternative) respiration 30% to the total respiration rate. Apperent K_m values of cytochrome oxidase in all cases were lower than those obtained for the alternative oxidase.

Differences in critical oxygen pressure for respiration (COPR) and in apparent K_m for oxygen, however, were strongly correlated with differences in root porosity and root diameter. K_m values at high and low temperatures were determined and from Arrhenius plots of oxygen uptake rates between 11° and 32°C, the role of diffusional impedance could be estimated. Root respiration of *R. maritimus* and *R. crispus,* both with high root porosity, but differing in root diameter, had a low K_m for oxygen (3 to 7 µM).
Contrasting with this were the responses, found for *R. thyrsiflorus* with thin roots, but low root porosity: a high K_m (10 to 20 µM) was found at all temperatures. The role of diffusional impedance as a function of temperature in oxygen uptake rate by the three species is discussed in relation to the differential resistance of the species towards flooding.

INTRODUCTION

In plant species, occurring on waterlogged soils, hypoxic conditions in the root environment are soon reached and under these conditions an efficient oxygen uptake can be decisive for growth and survival of roots.

The oxygen gradient from the outer towards the inner root cells depends mainly on root diameter, root porosity and respiratory activity of the root cells (Armstrong 1979; Van Noordwijk & De Willigen 1984; Armstrong & Beckett 1985). Thin roots are advantageous under hypoxia, since in them the effect of an oxygen gradient from the outer to the inner root zone is minimal, and anoxia in the stele is postponed to the utmost (Bowling

Abbreviations: SHAM, salicyl hydroxamic acid; MES, 2-N-morpholino ethane sulphonic acid; COPR, critical oxygen pressure for respiration

1973; De Boer & Prins 1984; De Willigen & Van Noordwijk 1984; Armstrong & Beckett 1985). A high root porosity or the formation of aerenchyma can considerably reduce diffusive resistance, and thus will help prevent the occurrence of anaerobic cores (Armstrong 1979; Armstrong & Beckett 1985; Van Noordwijk & De Willigen 1984). Consequently, diameter and porosity will determine to a large extent the oxygen gradient characteristics within the root.

Oxygen uptake in roots occurs mainly by cytochrome oxidase, with a high affinity for oxygen (Hayaishi 1962; Lambers 1985) and the alternative oxidase, with a lower affinity for oxygen (Lambers *et al.* 1983; Lambers 1985; Møller *et al.* 1988). The alternative oxidase will therefore be the first to be inhibited by a developing hypoxia.

It has been suggested (Armstrong & Webb 1985; Armstrong & Beckett 1987; Atwell & Greenway 1987) that diffusional resistance rather than enzyme kinetics determines the Critical Oxygen Pressure for Respiration (COPR), i.e. the oxygen concentration, below which respiration is concentration-dependent (Berry & Norris 1949). This conclusion is mainly based on experiments performed at 20-25°C, but soil temperatures are generally much lower. The reaction rate of an enzymatic process is strongly dependent on temperature, while diffusion is only slightly influenced (Berry & Norris 1949; Ngo & Laidler 1978, Bienfait *et al.* 1983), and temperature may therefore determine to a large extent whether oxygen uptake of a particular root system is predominantly determined by diffusion or by enzyme kinetics.

We investigated whether differences in root anatomy of three aerobically grown *Rumex* species give rise to different oxygen uptake characteristics. These *Rumex* species occur in the river ecosystem in the Netherlands, there being confronted with transient flooding. The investigated species differ in their flood-tolerance mainly because the tolerant species develop a new, aerenchymatous root system (Laan *et al.* 1989), which can relieve the oxygen stress by means of internal longitudinal oxygen diffusion (Laan *et al.* 1990). Nevertheless, the formation of these new roots takes 3-7 days (Chapter 7, this thesis) and

the already existing roots of both tolerant and intolerant *Rumex* species have to overcome the intermediate period. Survival then depends on the capacity of the aerobically grown root systems to get hold of oxygen at low oxygen concentrations.

COPR-values of the aerobically grown root systems and apparent K_m-values for cytochrome- and alternative oxidase were derived from oxygen depletion curves. With the use of Arrhenius plots, oxygen uptake kinetics of the three species were compared at different temperatures and related to kinetics of the respiratory system and to anatomical characteristics, i.e. root diameter and root porosity.

MATERIALS AND METHODS

Plant growth

Seeds of *Rumex thyrsiflorus* Fingerh., *R. crispus* L. and *R. maritimus* L. were collected from natural populations and sown on black polyethylene grains (Stamylan LD, DSM, The Netherlands) in nutrient solution (Laan *et al.* 1990). After germination they were transferred to polyethylene containers (8 l) and allowed to grow for 3-5 weeks on aerated nutrient solution in a growth room (temp. 22°C; 16 h light at 200 µmol $m^{-2}s^{-1}$; 8 h dark). The nutrient solution was changed twice a week.

Root respiration and determination of COPR-values

The depletion of oxygen from the root environment was measured in nutrient solution plus 1% (w:v) glucose on whole root systems (including the tap-root) in a system described before (Laan *et al.* 1990). This system consists of a 179-ml thermostatted cuvette, closed by a 'Perspex' lid with a 0.8 mm hole in the middle. The temperature was kept constant at 25.0°C with a thermostatted waterbath (Haake G-D8). The zero-oxygen level was calibrated by bubbling the nutrient solution with nitrogen gas until the reading was constant, and checked by Winkler-titration.

For determination of root respiration at different temperatures, the oxygen uptake by whole root systems (including the tap-root) and by yeast cells was again followed in nutrient solution plus 1% (w:v) glucose. After each depletion curve the root system was transferred to aerated nutrient solution (room temperature) and thoroughly rinsed with demineralised water. Oxygen uptake of single root systems and of yeast cells, starting at maximal oxygen concentration until total depletion of oxygen from the medium, was determined at 32.0, 25.0, 18.0 and 11.0 or 12.0°C, starting with the highest temperature. At the end of each temperature series, a second depletion curve at 32°C was made to check for loss of respiratory activity; this loss never exceeded 5% and no corrections were made. The calculation of root respiration was based on the fresh weight of the lateral root system only, since the contribution of the tap-root to total root weight differs between the species but hardly contributes to respiration (< 10%, P. Laan, unpublished results).

Determination of the activity of cytochrome- and alternative respiration path

The *in vivo* capacity and engagement of the alternative respiration pathway of the root systems of the *Rumex* plants, was determined by the addition of SHAM to the nutrient solution in the root vessel with a syringe. Iron was left out from the nutrient solution, since it is chelated by SHAM (Lambers 1980). SHAM was dissolved in 2-methoxy-ethanol and the maximal volume added was 1 ml; this amount of 2-methoxy-ethanol had no effect on root respiration. Cytochrome-coupled respiration was inhibited with KCN. To prevent a rise in pH, in these experiments buffer was added (20 mM MES, pH 6.0).

To determine the concentration of SHAM, needed to fully inhibit the alternative pathway, oxygen uptake was titrated with SHAM in the absence and presence of KCN (Fig. 1a). In most cases total inhibition was reached at 1 mM SHAM and, hence in subsequent experiments 2 mM SHAM was used. When a set of respiration data, obtained with different concentrations of SHAM, in the absence of KCN, is plotted against a similar set of data in the presence of KCN, the engagement of the alternative path can be derived from the slope of the line (Lambers 1985). In all root systems used, the alternative pathway was fully engaged (slope 1.0, Fig. 1b).

The cytochrome pathway was totally inhibited at 0.5 mM KCN (Fig. 1c) and in the experiments 1 mM KCN was used.

Determination of 'apparent' K_m-values

As respiration depletion plots do not yield the actual K_m value of the respiratory enzyme systems for oxygen, the derived values are described as 'apparent K_m'. Apparent K_m-values for oxygen of cytochrome oxidase-dependent respiration were derived from single depletion profiles in the presence of 2 mM SHAM, those of alternative oxidase-dependent respiration from single depletion plots in the presence of 1 mM KCN.

In Arrhenius plots, apparent K_m values were determined by halving the log-transformed maximal respiration rates.

Root diameter and root porosity

Diameters of randomly chosen roots of aerobically grown *Rumex* plants were determined at 1 cm behind the root apex with a stereo-microscope. Porosity of the same roots (0.3-1 g FW) was determined by pycnometry (Laan *et al.* 1989).

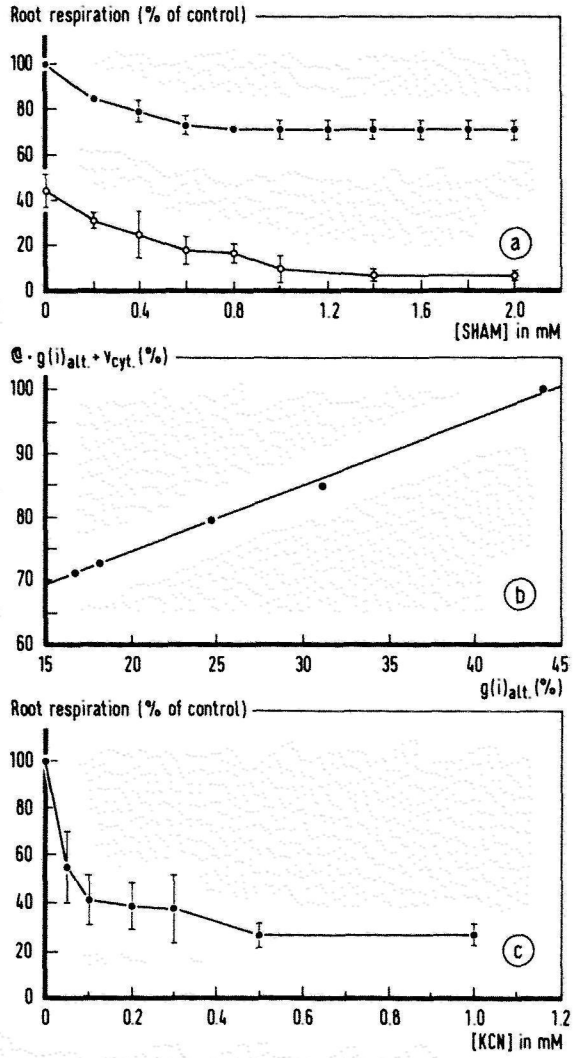

Figure 1 **(a)** Titration of root respiration of *R. maritimus* with SHAM (●), and with SHAM plus 1 mM KCN (○), (means of 3 replicates ± SD; 100% respiration was 213 ± 5 µmol O_2 g^{-1}DW h^{-1}); **(b)** Root respiration at different concentrations of SHAM in the absence of KCN as a function of a similar set of values obtained in the presence of 1 mM KCN (data from Fig. 1a). The slope of the lineair regression line ($y = 1.04x + 53.8$; $r^2=0.996$), designates the fraction of the alternative path which is engaged; **(c)** Titration of root respiration of *R. maritimus* with KCN (means of 3 replicates ± SD; 100% respiration was 144 ± 14 µmol O_2 g^{-1}DW h^{-1})

Root respiration and engagement of different respiration pathways

The *in vivo* contribution of the different pathways to respiration varied only slightly between the *Rumex* species: cytochrome-dependent respiration amounted to 65-70% of total respiration, alternative respiration to 30-35% (Table 1). The contribution of residual respiration was low in all cases (3-5% of total, see Fig. 1a).

From the single depletion plots, COPR- and apparent K_m values for cytochrome- and alternative oxidase were derived at 25°C (Table 2). COPR values of *R. thyrsiflorus* were significantly higher than those of *R. crispus* and *R. maritimus*, the COPR for yeast was considerably lower.

In all species, the apparent K_m of the alternative oxidase-dependent respiration was higher than that of cytochrome oxidase-dependent respiration (Table 2). The contribution of cytochrome and alternative pathways to total respiration was the same at 25° and 11°C (Table 1)

Table 1 The activity of respiration pathways of intact root systems of *Rumex* species at high and low temperatures (means of 4 replicates ± SD (25°C) or single measurements (11°C); data as a percentage of total respiration, based on inhibition of the different pathways with 2 mM SHAM or 1 mM KCN, see Fig. 1; 100% respiration was (means ± SD): 103 ± 10 *(R. thyrsiflorus)*, 124 ± 20 *(R. crispus)* and 206 ± 45 *(R. maritimus)* μmol O_2 g^{-1}DW h^{-1})

	cytochrome path	alternative path	
Species	25°C	25°C	11°C
R. thyrsiflorus	69 ± 2	31 ± 2	31
R. crispus	65 ± 1	35 ± 1	24
R. maritimus	70 ± 4	30 ± 3	24

Table 2 Critical oxygen pressures for respiration (COPR) and apparent K_m-values for oxygen of cytochrome and alternative oxidase of roots of *Rumex* species and of yeast at 25°C (in µM O_2; means of 6 (COPR) or 3 replicates ± SD; 100% respiration was (means ± SD): 103 ± 10 *(R. thyrsiflorus)*, 124 ± 20 *(R. crispus)*, 206 ± 45 *(R. maritimus)* µmol O_2 g^{-1}DW h^{-1} (COPR and 'K_m cytochrome oxidase'); *R. thyrsiflorus* 124 ± 16, *R. crispus* 193 ± 77, *R. maritimus* 162 ± 3 µmol O_2 g^{-1}DW h^{-1}('K_m alternative oxidase')

Species	COPR	apparent K_m	
		cytochrome oxidase	alternative oxidase
R. thyrsiflorus	49 ± 10	10 ± 1	18 ± 2
R. crispus	31 ± 5	6 ± 1	15 ± 1
R. maritimus	27 ± 9	6 ± 1	26 ± 1
Saccharomyces cerevisiae	13 ± 1	4 ± 1	-

Arrhenius plots

As temperature strongly influences reaction rate, but has much less effect on diffusion, the oxygen uptake rates of the root systems at different temperatures may give insight into the relative importance of diffusive resistances and enzyme kinetics (Berry & Norris 1949; Ngo & Laidler 1978; Bienfait *et al.* 1983).

Yeast was used as a model system. Oxygen depletion curves obtained with yeast suspensions revealed that the lowest oxygen concentration, which could be reliably measured was 1 µM, and the K_m obtained from the largely parallel curves was 2 µM (Fig. 2a). Typically, this value did not vary with temperature. By embedding yeast in 10% agar, an artificial diffusion barrier was added. This resulted in an Arrhenius plot with lines diverging from low to high temperatures (Fig. 2b). As a consequence, K_m varied between ca. 4 µM at 12°C and 10 µM at 32°C.

With the root systems, an oxygen uptake pattern resembling that of agar-embedded yeast was found in *R. thyrsiflorus* (Fig. 2c): oxygen concentration lines diverged from low to high temperatures, resulting in an increase of K_m with increasing temperature, from 10 µM at 11°C to 20 µM at 32°C (Fig. 3c).

Rumex maritimus roots showed a temperature-dependent oxygen uptake pattern, which

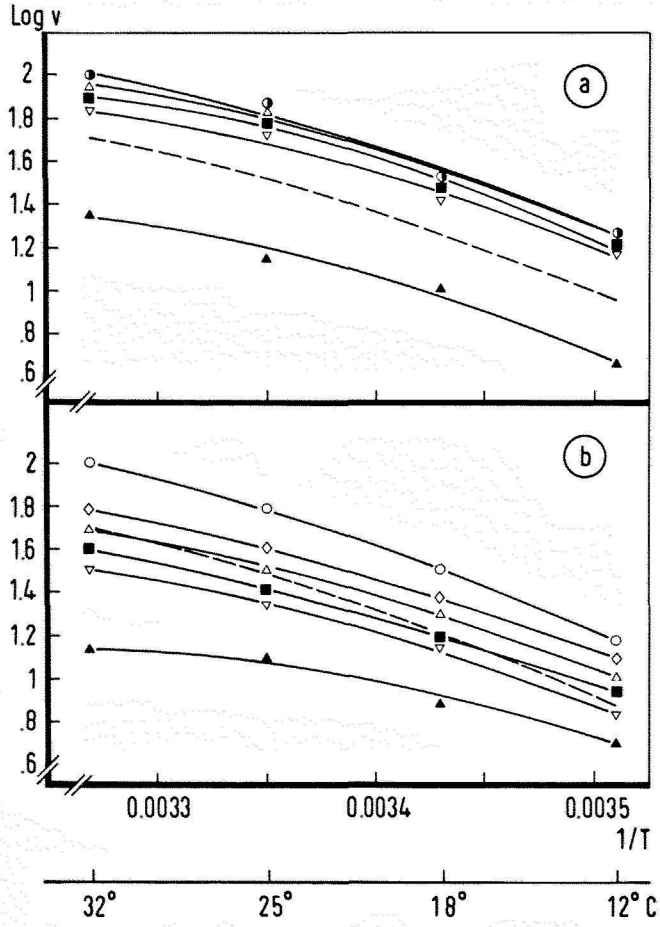

Figure 2 Arrhenius plots for oxygen uptake by yeast *(Saccharomyces cerevisiae)* in free suspension **(a)**, and embedded in 10% agar **(b)** at different solution oxygen concentrations: 1 µM (▲), 3 µM (▽), 5 µM (■), 10 µM (△), 20 µM (◊), 30 µM (◑) and 100-150 µM O_2 (=maximum)(○); K_m as a dotted line. Data are means of 2 replicates; oxygen uptake rates as a percentage of maximum rate at 32°C, which were: 44.3 µmol O_2 ml^{-1}(yeast suspension) h^{-1}, and 13.5 µmol O_2 g^{-1}(yeast in agar) h^{-1}, respectively)

closely resembled that of yeast in suspension, i.e. practically parallel lines in the Arrhenius plots and thus, a temperature-independent K_m value of 5 µM (Fig. 3a). This K_m value was two (at 11°C) to four (at 32°C) times lower than that found for *R. thyrsiflorus.*

Roots of *R. crispus* showed an intermediate pattern: at lower temperatures, the lines were parallel as with *R. maritimus;* at higher temperatures, the lines diverged as with

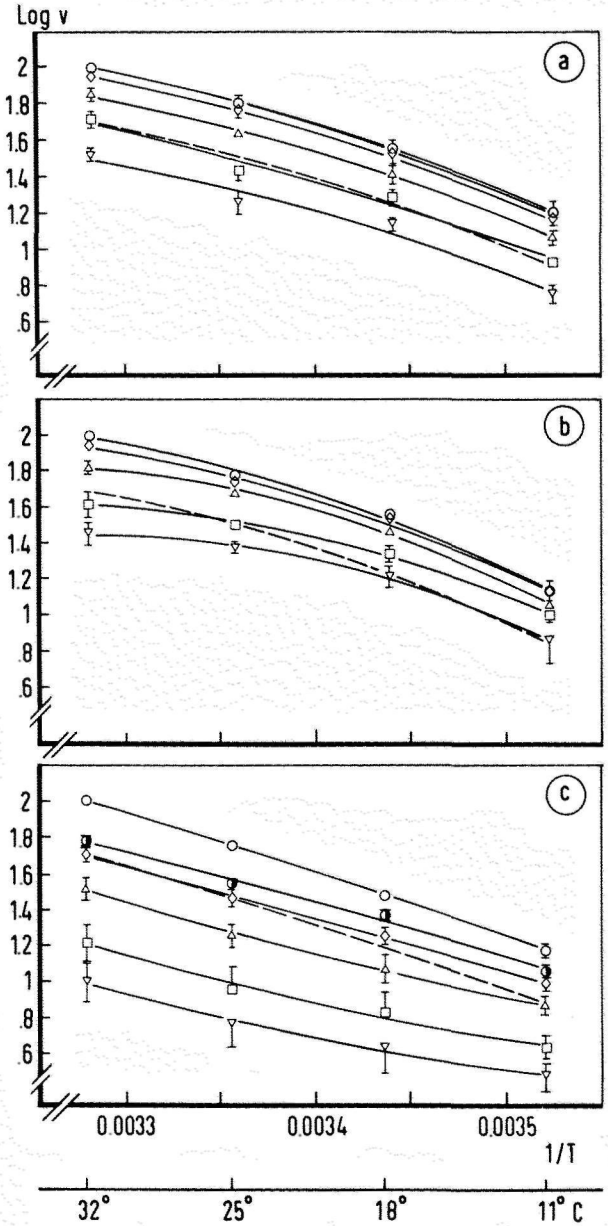

Figure 3 Arrhenius plots for oxygen uptake by intact root systems of *Rumex maritimus* **(a)**, *R. crispus* **(b)** and *R. thyrsiflorus* **(c)** at different solution oxygen concentrations: 3 µM (▽), 5 µM (□), 10 µM (△), 20 µM (◇), 30 µM (◑) and 100-150 µM O_2 (= maximum) (○); K_m as a dotted line (means of 3 replicates ± SE; data as percentage of maximum respiration rate of lateral roots at 32°C, which were: *R. thyrsiflorus* 81 ± 13, *R. crispus* 27 ± 8, and *R. maritimus* 39 ± 8 µmol O_2 g^{-1}FW h^{-1}, respectively)

R. thyrsiflorus. The apparent K_m therefore increased with increasing temperature from ca. 3 µM at 11°C to ca. 7 µM at 32°C (Fig. 3b). These K_m-values were in the same range as for *R. maritimus* and at all temperatures three times lower than for *R. thyrsiflorus*.

The lines might also be presented as straight lines with a 'bending point' (cf. Berry & Norris 1949; Ngo & Laidler 1978; Bienfait *et al.* 1983). In a well defined system with a precisely known location of enzyme activity and of diffusion paths this may be justified. In the *Rumex* root systems with their heterogeneous cell layers, all contributing to respiration but to different extents (Armstrong & Beckett 1985, 1987) and different degrees of porosity within the tissue, a more gradual change in the slopes of the lines in the Arrhenius plots is to be expected. The same holds for the yeast blocks with diameters varying between 0.5 and 1.5 mm. For the sake of comparison, all lines were therefore fitted as second-order polynomes.

Root anatomy

Clearcut differences in both root diameter and root porosity between the species were observed: *R. thyrsiflorus* had the thinnest roots and lowest porosity, *R. maritimus* and *R. crispus* roots had high porosities, the roots of *R. crispus* being thickest (Table 3).

Table 3 Root porosity and root diameter of laterals of *Rumex* species (porosity of root segments 8-10 cm behind the apex, diameter at 2 cm behind the apex; means of 3 (porosity) or 30 (diameter) replicates ± SD)

	Root diameter (mm)	Root porosity (% v:v)
R. thyrsiflorus	0.38 ± 0.00	7.6 ± 1.7
R. crispus	0.65 ± 0.09	20.6 ± 1.9
R. maritimus	0.43 ± 0.06	19.3 ± 3.0

DISCUSSION

The results of this study show that roots of closely related plant species may have very different respiration characteristics. These differences might be caused by varying degrees at which oxygen consuming enzymes contribute to respiration. In the three *Rumex* species studied, no such differences were observed: cytochrome oxidase with high and alternative oxidase with low affinity for oxygen, in all cases contributed for 70 and 30% respectively to respiration. This was the case at both high and low temperatures (Table 1).

On the other hand, the three species showed considerable differences in root anatomy (Table 3), implying large differences in diffusion-path length for external oxygen to respiring cells. Experiments with yeast in free suspension and in agar were performed in order to determine the effect of diffusional resistance, at dimensions comparable to those in *Rumex* roots.

The Arrhenius plot obtained with roots of *R. maritimus* was comparable to that of yeast in free suspension, resulting in a low K_m, uninfluenced by temperature. *R. crispus* showed, at lower temperatures, the highest affinity for oxygen, but the lines in the Arrhenius plot diverged, indicating a lower affinity for oxygen at higher temperatures. In the Arrhenius plot for *R. thyrsiflorus* the lines were at much larger distances than in the other two species, which implies a strong influence of diffusion on oxygen uptake at all temperatures.

Our explanation of these results is as follows. Both *R. maritimus* and *R. crispus* have a high root porosity, leading to a low diffusional resistance for oxygen to the respiring cells, even those in the stelar zone. *R. crispus* has thicker roots and, thus the diffusion pathway for oxygen to the stelar cells is longer than in *R. maritimus;* this explains the more important role of diffusion at higher temperatures in *R. crispus.* At the lower temperatures, a K_m even lower than in *R. maritimus* was obtained (Fig. 3). This low K_m reflects the combined effect of easy diffusion and low respiration rate of the roots (27 μmol O_2 g^{-1}FW h^{-1} for *R. crispus* and 39 μmol O_2 g^{-1}FW h^{-1} for *R. maritimus,* Fig. 3).

R. thyrsiflorus has the thinnest roots of the three species, but its root porosity is very low, so that diffusion is the most important limiting factor for respiration throughout the temperature range studied. But even then more so at the higher temperatures.

In the field, *R. maritimus* is found at the lowest places, which are frequently flooded, *R. thyrsiflorus* grows at much higher elevation at places which are seldomly flooded and *R. crispus* is intermediate (Laan *et al.* 1989; Blom *et al.* 1990).

We conclude that the oxygen efficiency of primary, aerobically grown roots, as affected by respiration rate, root porosity and root diameter, is higher in those species which are most often subjected to low oxygen concentrations in the soil. This is important in situations of transient flooding or in compacted soils, when hypoxic and not yet anoxic conditions prevail. At the moment that oxygen in the soil is completely depleted, the newly formed, aerenchymatous roots must relieve the oxygen stress of the root system of the low-elevated species via internal longitudinal oxygen transport (Laan *et al.* 1990).

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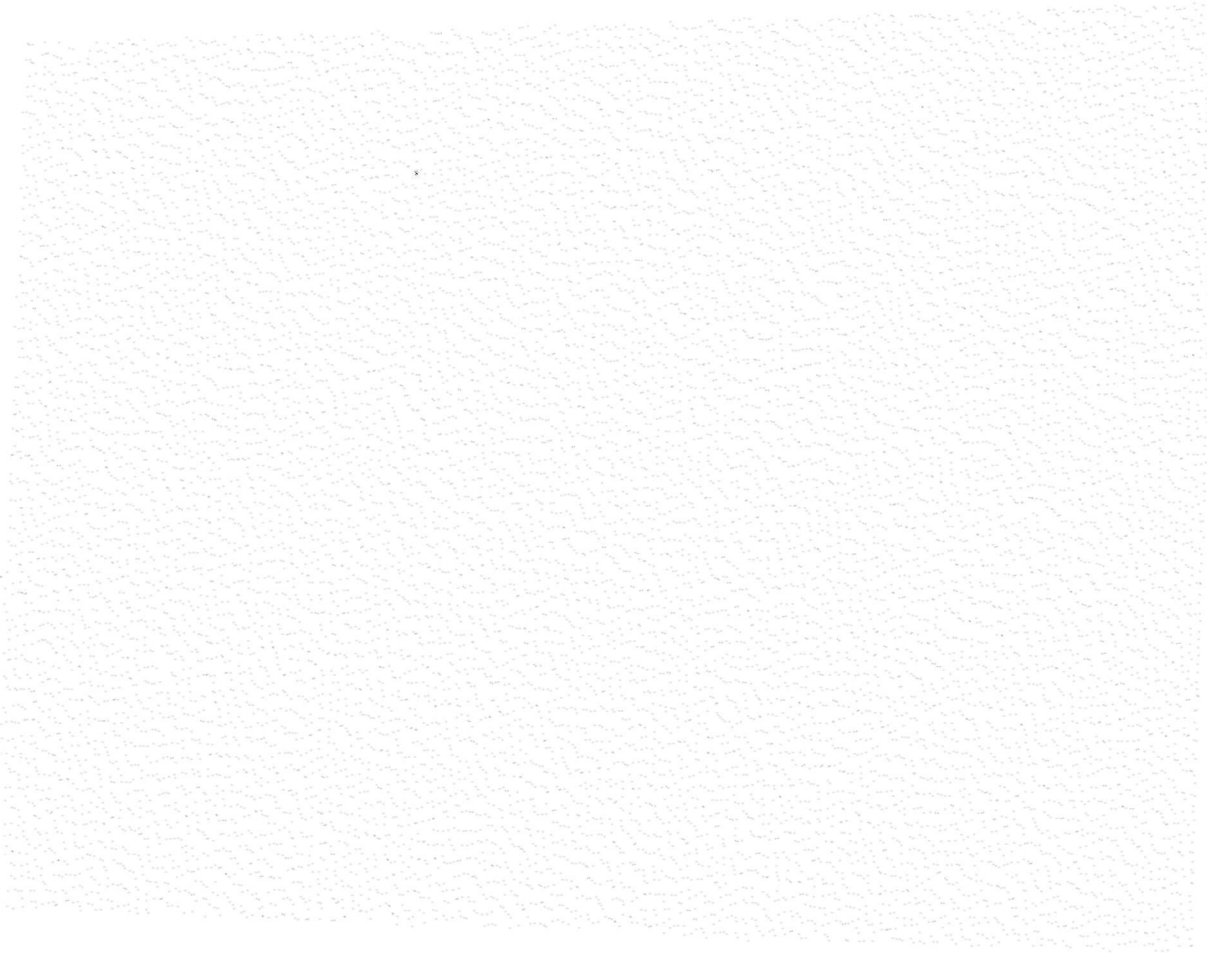

CHAPTER 6 Growth and survival responses of *Rumex* species to flooded and submerged conditions: the importance of shoot elongation, underwater photosynthesis and reserve carbohydrates

with C.W.P.M. Blom
Journal of Experimental Botany (1990) (in press) (with permission)

CHAPTER 7 Growth and development of *Rumex* roots as affected by hypoxic and anoxic conditions

with J.M.A.M. Clement & C.W.P.M. Blom

GROWTH AND SURVIVAL RESPONSES OF *RUMEX* SPECIES TO FLOODED AND SUBMERGED CONDITIONS: THE IMPORTANCE OF SHOOT ELONGATION, UNDERWATER PHOTOSYNTHESIS AND RESERVE CARBOHYDRATES

SUMMARY

Plants of *Rumex thyrsiflorus* Fingerh., *R. crispus* L. and *R. maritimus* L., which are zoned along a gradient of elevation in a river foreland ecosystem, and differ in their flood-tolerance, were subjected to different flooding levels. Under conditions of soil flooding, the growth rates of the flood-tolerant *R. crispus* and *R. maritimus* were as high as under drained conditions, but that of the flood-intolerant *R. thyrsiflorus* was halved. Upon submergence, the low elevation species *R. maritimus* showed rapid shoot elongation; when elongation resulted in a protrusion of leaves above the water surface, the plants survived. Alternatively, underwater photosynthesis also led to a 100% survival of submerged *R. maritimus* plants, provided that enough inorganic carbon was made available in the water. This could be attributed in part to the use of photosyntetically derived oxygen for root respiration; in a hydroculture experiment, with 5 mM CO_2 in the water in the shoot environment, photosynthetically derived oxygen contributed more than 50% to root oxygen consumption at low oxygen concentrations in the root environment.

The intermediately elevated species *R. crispus* appeared to be much more tolerant towards conditions of prolonged total submergence: older plants survived an eight weeks submergence treatment in the dark. This response was explicable in terms of a dormancy-strategy, which is supposed to be characterized by a slow consumption of carbohydrates stored in the tap-root. The differential responses of *R. maritimus* and *R. crispus* to total submergence reveal the limitations of flood-tolerance and reflect the different life-histories of the species.

INTRODUCTION

Growth rate and biomass production of terrestrial plants under flooded conditions are normally reduced (Trought & Drew 1980; Drew 1983; Jackson & Drew 1984). Wetland species, however, are able to cope with such conditions because of the formation of an aerenchyma system, which provides an efficient means to maintain aerobiosis in the root system (Armstrong 1979; Laan *et al.* 1989b; Laan *et al.* 1990) and as a consequence, growth can be maintained or restored.

In *Rumex* species, the development of aerenchyma determines to large extent the degree of tolerance to flooding (Laan *et al.* 1989a). However, under conditions of total

submergence, a phenomenon which sometimes takes place during the growing season of flood-tolerant *R. maritimus* and *R. crispus* plants in their natural habitat, i.e. the river foreland ecosystem (Blom 1990; Blom *et al.* 1990), this 'avoidance strategy' fails, because the free diffusion or convection pathway for oxygen between air and root system is blocked.

Under such conditions a number of phenomena may take place to overcome or compensate for the lack of oxygen: (i) petiole and leaf elongation by which leaves reach the water surface after which aerobic conditions in the root system can be restored (Atwell *et al.* 1982; Barclay & Crawford 1982; Keith *et al.* 1986; Brändle 1990; Laan *et al.* 1990), (ii) underwater photosynthesis to restore both sugar and oxygen supply to the roots (Bowes 1987; Gaynard & Armstrong 1987; Setter *et al.* 1987), (iii) dormancy of the plant (Barclay & Crawford 1982, 1983; Brändle 1985), (iv) anaerobic respiration, demanding a high sugar supply (Crawford 1982; Jackson & Drew 1984; Setter *et al.* 1987; Brändle 1990).

In this study, growth and survival responses of *Rumex maritimus*, *R. crispus* and *R. thyrsiflorus* upon soil flooding and upon total submergence were examined. Growth rates after 3 and 6 weeks of soil flooding or total submergence of *R. thyrsiflorus, R. crispus* and *R. maritimus* were determined. Survival responses and mortality rates of the flood-tolerant *R. maritimus* and *R. crispus* after different periods of total submergence were related to factors that may determine their differential flood-tolerance, such as shoot elongation, underwater photosynthesis, and the content of carbohydrate reserves in the tap-root.

MATERIALS AND METHODS

Plant growth

Seeds of *Rumex maritimus* L., *R. crispus* L. and *R. thyrsiflorus* Fingerh., collected from natural populations in the river area near Nijmegen (The Netherlands), were sown on black polyethylene grains in a germination cell (16 h light at 60 µmol $m^{-2}s^{-1}$ (PAR), 25°C; 8 h dark, 15°C). After germination, the seedlings were transplanted to PVC tubes (length 0.40 m, diameter 0.12 m), filled with a clay/sand mixture (1:1 (v:v) air-dried and homogenized

clay from a beet-field (pH(H_2O) 6.9, weight percentage organic matter 5.4 ± 0.1) and cleaned, air-dried sand), and allowed to grow for several weeks in a greenhouse (16 h light at a minimum intensity of 200 µmol $m^{-2}s^{-1}$ and a maximum of 1200 µmol $m^{-2}s^{-1}$ (PAR), 15-22°C). The tubes were watered three times a week to field-capacity. Plants of several different ages were used for the experiments. In the flooding treatments, the tubes were placed in an open container (1 x 1 x 1 m) and the water was raised to 1-2 cm above the soil surface. In the total submergence treatments, tubes were also placed in an open container, and the plants were inundated with tap-water to 0.55 m above the tubes. Fine wire-netting was placed on the top of the containers to prevent the leaves from protruding above the water surface. During the submergence treatment light intensity was measured at different depths (LI-COR photometer; LI-185B, Lambda Instr. Corp., USA) and every 5 or 6 days the water was gradually replaced. This prevented the plants from being exposed to the air. Control plants were watered daily to field capacity. Plants were harvested by pushing out the soil core with the complete root system. The root system was separated from the soil by rinsing the soil on a sieve. Shoots, tap-roots and laterals were separated and fresh weight was determined. Dry weights were determined after 48 h drying at 70°C.

Mortality was determined at the end of the experiment by placing the plants under drained conditions for two weeks. When growth of old leaves and/or formation of new leaves did not occur within this period, the plants were considered dead.

Starch content of tap-roots

Tap-roots were ground with a grinding mill (Cyclotec 1093 Sample Mill, Tecator, Sweden). Ca. 50 mg of dry tap-root material was suspended in 2 cm^3 of demineralized water, autoclaved (15 min, 121°C) and, after cooling to 37°C, incubated for 24 h with 2 cm^3 amyloglucosidase-solution (1.5 cm^3 amyloglucosidase (EC 3.2.1.3) of specific activity 75 U mg^{-1} protein (Sigma Chem. Comp., USA) in 100 cm^3 of a 100 mol m^{-3} acetate buffer, pH 4.5) at 37°C in a shaking water-bath. The suspension was carefully adjusted to exactly 50 cm^3 and centrifuged (49,500 g for 20 min). Glucose was determined in the supernatant with the Anthrone-method (Yemm & Willis 1954).

Photosynthesis and inorganic carbon content of the water

Photosynthetic activity of six totally submerged plants of each species was estimated by trapping gas escaping from the leaves via a funnel attached to a 25 cm^3 pipette at least for 3 h at a constant light intensity at plant level of 160-175 µmol $m^{-2}s^{-1}$ (temperature 20-23°C). The pH of the water varied with time and place from 8.0-9.5 (containers with tap-water), or from 8.3-8.9 (containers with tap-water, enriched with 10 mM $NaHCO_3$). The volume of the entrapped gas was recorded every 30 min. Gas release from plants under dark conditions was used as reference.

Total leaf area was determined with a leaf area meter (LICOR 3000, Lambda Instr. Corp., USA), or in case leaf area was less than 0.3 m^2, with a 'magnetic board-area-meter' (MOP Kontron system, GMBH, München, W. Germany, accuracy 5 mm^2).

The bicarbonate content of the water was determined during photosynthetic measurements by titration of water-samples with 0.1 N HCl to pH 4.2.

Quantification of photosynthetic oxygen used for root respiration

Plants of *R. maritimus* were grown on aerated hydroculture for four weeks in a nutrient solution consisting of: Macro-nutrients: 1 mol m^{-3} KNO_3, 1 mol m^{-3} $Ca(NO_3)_2$, 1 mol m^{-3}

$NaNO_3$, 0.5 mol m^{-3} KH_2PO_4, 0.25 mol m^{-3} $MgSO_4$; Micro-nutrients: 0.25 mol m^{-3} FeEDTA, 12.5 mmol m^{-3} KCl, 6.3 mmol m^{-3} H_3BO_3, 0.5 mmol m^{-3} $MnSO_4$, 0.5 mmol m^{-3} $ZnSO_4$, 0.1 mmol m^{-3} $CuSO_4$, 0.1 mmol m^{-3} H_2MoO_4, and transferred to a stagnant 0.05% (v:v) anaerobic agar in nutrient solution. After one week of anaerobiosis, a large number of new aerenchymatous roots had developed. These plants were used for root respiration measurements in a previously described system (Laan *et al.* 1990). This system consists of a thermostatted root compartment (volume 1.8 * 10^{-4} m^3), and a perspex shoot compartment. The root compartment contained the above-mentioned nutrient solution; the shoot compartment was slowly filled with a 0.05 M NaH_2PO_4 buffer solution (pH 4.95), until total submergence of the shoot (approx. 3.5 * 10^{-3} m^{-3}) and stirred with a magnetic stirrer. The depletion of oxygen was followed in the root compartment with the same plant under dark and under light conditions in the shoot compartment. First, a fully aerated nutrient solution was circulated several times through the root vessel, until an oxygen concentration of ca. 220 µM was reached. Then the root compartment was closed and the depletion of oxygen was followed. The first depletion curve was always made with the shoot in the dark by blinding the perspex tube with a black plastic cover. When oxygen was completely depleted from the root compartment, the nutrient solution was replaced by circulating fully aerated nutrient solution through the root vessel; the black cover was removed and the shoot illuminated (150 W Leitz Wetzlar photolamps, one at the top, the other at the side of the perspex tube, giving a mean light intensity of ca. 600 µmol $m^{-2}s^{-1}$ PAR; temperature of the water 24°C). Sodium bicarbonate was added to the buffer solution in the shoot compartment, giving a final concentration of 5 * 10^{-4} M bicarbonate (at this pH almost completely as CO_2). After 10-15 min the root vessel was closed and again, the depletion of oxygen from the root compartment was followed. Following the same procedure, the third depletion curve was made after an extra addition of bicarbonate, giving a final concentration of 5 * 10^{-3} M in the shoot compartment. At the end of each series of measurements the shoot was cut off and sealed with a mixture of clay and plasticine, after which another depletion curve was made.

The contribution of photosynthetically-derived oxygen to root respiration was calculated by subtracting the root oxygen uptake rate of the plant with the shoot in the light plus additional bicarbonate from the root oxygen uptake rate (at the same oxygen concentration in the root environment) of the plant with the shoot in the dark. This was done at different root oxygen concentrations down to COPR (see for further details Laan *et al.* 1990).

Photosynthesis of leaf discs

The specific photosynthetic activity of leaf discs (diameter 3.5 cm) of *R. maritimus* and *R. crispus* was determined in the system described above. Instead of a Perspex tube, the cuvette was sealed with a double-walled transparant Perspex lid; the internal solution consisted of 0.05 M NaH_2PO_4 buffer in nutrient solution either at a pH of 8.0 or at pH 4.95. The solution was kept at 25.0°C by a flow-through of thermostatted water via both outer compartment of the cuvette and cooling compartment of the Perspex lid. A Leitz Wetzlar photolamp was mounted above the cuvette, giving a light intensity at the discs of 600 µmol $m^{-2}s^{-1}$. Discs were cut from leaves of the same age and mounted on a perforated table of the same dimensions with a fine copper wire. Via a central hole in the middle of the Perspex lid, different amounts of $NaHCO_3$ solution were added to the buffer solution. The production of oxygen at different bicarbonate (at pH 8.0) and at different CO_2-equivalents (at pH 4.95) was recorded with an oxygen electrode up to a CO_2- concentration at which saturation of photosynthesis took place. At the end of each measurement series, the cuvette was darkened with a black plastic cover and, dark respiration was determined.

Growth rates, biomass production and survival under soil-flooded and submerged conditions

The relative growth rates (RGR) of the *Rumex* species after three and six weeks drainage, soil flooding and total submergence were compared (Table 1). In all species growth rate was lowered under flooded conditions, but the reduction was least severe in *R. maritimus*. After approx. 5 weeks, *R. maritimus* (under long-day conditions an annual species), formed a flower-stalk under both drained and flooded conditions. Consequently, RGR was reduced significantly, but this reduction was more or less the same under flooded and drained conditions. In *R. crispus* and *R. thyrsiflorus*, RGR was halved upon flooding in the first 3 weeks; in the ensuing period, RGR again was half as high under flooded than under drained conditions in *R. thyrsiflorus* (32 vs. 67 mg g^{-1}DW, but only slightly affected in *R. crispus* (54 vs. 64 mg g^{-1}DW, Table 1).

When totally submerged, only *R. crispus* was able to maintain biomass throughout the first 3 week-period (-1 ± 3 mg g^{-1}DW day^{-1}, Table 1). *Rumex maritimus* started a fast elongation of leaves and leaf petioles, but after 10 days, all plants collapsed, since the

Table 1 Growth of *Rumex* species under drained, flooded or totally submerged conditions for 6 weeks and mortality after 3 and 6 weeks of total submergence (mean plant RGR of 6 replicates ± SE; age of the plants at the start of the treatment 4 weeks)

Species	Relative growth rate (mg g^{-1}DW day^{-1})				Mortality upon de-submergence (% of total)
	period (weeks)	drained	flooded	submerged	
R. thyrsiflorus	0-3	115 ± 5	66 ± 5	-22 ± 13	33
	3-6	67 ± 5	32 ± 7	-57 ± 11	100
R. crispus	0-3	150 ± 6	83 ± 6	-1 ± 3	0
	3-6	64 ± 3	54 ± 3	-58 ± 9	33
R. maritimus	0-3	126 ± 3	111 ± 4	-34 ± 6	100
	3-6	32 ± 2	41 ± 6	-91 ± 7	100

Table 2 Effect of 5 and 8 weeks of total submergence under light or dark conditions on leaf number, biomass production, starch content of the tap-root and recovery growth of *Rumex crispus* plants (means of 7 replicates ± SE; age of the plants at the start of the experiment 14 weeks; light intensity was a minimum of 150 μmol $m^{-2}s^{-1}$)

	5 weeks total submergence		8 weeks total submergence plus 2 weeks recovery growth	
	light	dark	light	dark
Number of green leaves	9.7 ± 0.7	4.1 ± 0.4	4.0 ± 0.8*	4.9 ± 0.7*
Biomass production				
Shoot, fresh weight (g)	68.2 ± 7.3	44.8 ± 4.3	8.4 ± 0.9*	5.8 ± 0.9*
dry weight (g)	4.0 ± 0.4	0.7 ± 0.1	2.8 ± 0.4*	0.4 ± 0.1*
Root dry weight				
tap-root (g)	4.3 ± 0.4	2.8 ± 0.5	3.9 ± 0.5	1.8 ± 0.3
lateral roots (g)	1.4 ± 0.1	0.8 ± 0.1	1.4 ± 0.1	1.1 ± 0.2
Tap-root starch content (mg glucose equivalents)				
Concentration				
(mg (g^{-1}DW)	693 ± 25	585 ± 43	670 ± 26	625 ± 27
Content per tap-root				
(mg)	3068 ± 342	1606 ± 271	2499 ± 413	1477 ± 201

* Newly formed shoot material only.

water surface had not been reached. Thus, under these conditions, *R. crispus* was most tolerant: after 6 weeks only one third of the plants had died, while all plants of the other two species failed to recover.

Older *R. crispus* plants appeared to be even more tolerant of total submergence (Table 2). An 8-week treatment in the dark did not kill any of the plants. Only a slight and transient leaf elongation took place, and the plants maintained biomass, possibly by slowing down their metabolism. In the light, more green leaves were maintained after 5 weeks of total submergence than in the dark (Table 2) and, as a consequence, the remaining biomass was higher than in the dark. Given that the total amount of reserve carbohydrates in roots was twice as high in light as in darkness (3068 vs. 1606 mg per tap-root, Table 2), it appears that underwater photosynthesis had contributed significantly to the sugar supply of the roots.

The typical response of *R. crispus* upon submergence seems therefore directed towards the maintainance of biomass, enabled by storage of sugars in the tap-root under drained conditions on the one hand, and possibly by a low consumption rate of this stored starch on the other.

In contrast, *R. maritimus* showed a fast shoot elongation of leaves and petioles upon submergence. There was a strong tendency to concentrate biomass in the youngest leaves, and to shed the older ones under both light and dark conditions. Lateral roots all died within 5 days (data not shown).

Conditions conducive to survival of submerged plants

The importance of shoot elongation in *R. maritimus,* which led to the protrusion of leaf tips above the water surface is shown in Figure 1. When the water was lowered sufficiently for the leaf tips to protrude only 7 cm, all plants survived. Biomass production of both tap-root and laterals was restored; the protruded part of a flower-stalk, which was formed underwater, flowered within a few weeks. On the other hand, when the water surface was not reached, the starch content in the tap-root, biomass of root and shoot parts decreased rapidly, and all plants died within four weeks.

The importance of underwater photosynthesis for growth and survival of young *R. crispus* and *R. maritimus* plants during submergence was estimated by comparison of biomass production, mortality rates and shoot elongation under dark and light with and without inorganic carbon added to the water (Table 3).

In the dark, shoot elongation was very poor in both species. *R. maritimus* showed a significant biomass reduction of both shoot and root. Leaves were shed and only 17% of the initial shoot biomass was left after 20 days; no laterals could be detected, and the tap-root declined to 74% of its initial biomass. These plants could not restore growth under drained conditions, and all died within 30 days (Table 3). In *R. crispus* there was also a biomass reduction, but this was less severe than in *R. maritimus.* After 20 days, most of the leaves remained viable, although a reduction of the initial shoot biomass was

recorded. A reduction of initial biomass was also found for the root system. However, all plants survived 20 and 30 days submergence treatments (Table 3).

In the absence of additional bicarbonate, light stimulated shoot elongation in *R. maritimus* (Table 3). Elongation was not associated with an increase in dry weight, but

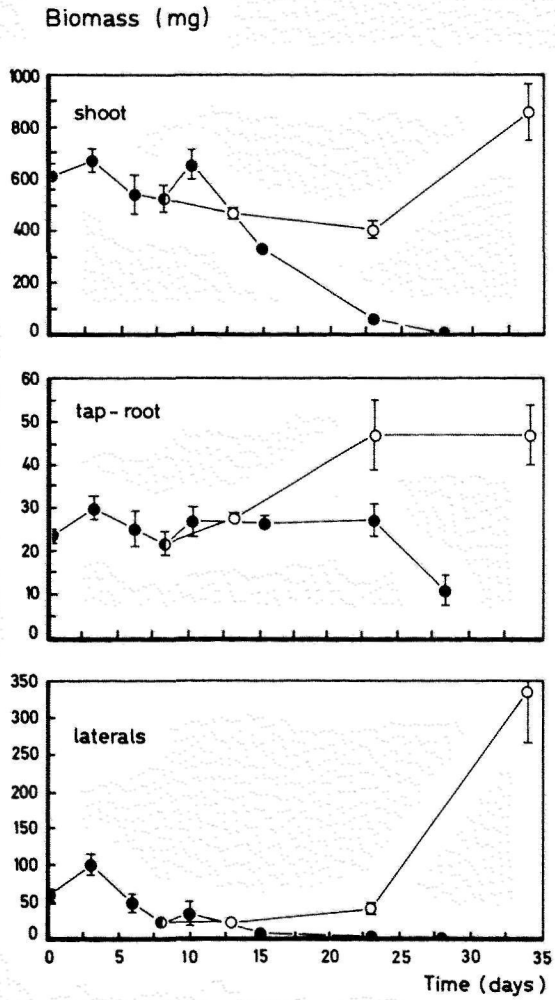

Figure 1 Biomass production of shoots, tap-roots and laterals of *R. maritimus* plants, which were continuously submerged (●), or were submerged for 8 days when the water level was lowered, so that leaf tips protruded above the water surface (○) (mean dry weights of 6 replicates ± SE; plants were 6 weeks old at the start of the treatment, light intensity at (submerged) plant level was 150-160 µmol $m^{-2}s^{-1}$ and inorganic carbon content of the water was ca. 1 mM)

Table 3 Effect of light and of addition of inorganic carbon to the water (light + C) on elongation and biomass production after 20 d, and mortality after 20 and 30 d of totally submerged *R. crispus* and *R. maritimus* plants.
Age of the plants at the start of the experiment 4 weeks; means of 10-13 (shoot height) or 6 (biomass production and mortality) replicates ± SE; height of the shoot at t = 0 (n=20±SE): *R. crispus* 17.7 ± 0.4, *R. maritimus* 18.7 ± 0.5 cm; biomass at the start of the experiment: *R. crispus:* shoot 439 ± 14, tap-root 36 ± 3, lateral roots 43 ± 6 mg DW; *R. maritimus:* shoot 611 ± 50, tap-root 23 ± 2, lateral roots 59 ± 8 mg DW; bicarbonate concentration: 1.35 mM (dark treatment), 0.98 mM (light treatment), and 10.9 mM (light + C)

Species	*R. crispus*			*R. maritimus*		
Treatment	dark	light	light + C	dark	light	light + C
Shoot height (cm)	21.1 ± 0.4	22.5 ± 0.3	33.6 ± 0.5	21.6 ± 0.3	34.6 ± 0.2	57.4 ± 0.4
Biomass (% of t=0)						
Tap-root	78 ± 11	125 ± 17	378 ± 17	74 ± 30	152 ± 26	235 ± 13
Lateral roots	65 ± 12	91 ± 21	188 ± 19	0	10 ± 2	120 ± 12
Shoot	62 ± 5	89 ± 10	297 ± 30	17 ± 3	37 ± 7	157 ± 17
Mortality (%)						
-after 20 days	0	0	0	100	0	0
-after 30 days	0	0	0	100	100	0

increased water uptake fully accounted for the increase in fresh weight of the shoot. Although less severe than under dark, submerged conditions, the effect of light was to decrease the biomass of shoots and lateral roots (to 10 and 37% of initial biomass respectively, Table 3); only the tap-roots increased their biomass. All plants survived a 20 days submergence period, but after another 10 days they were dead.

In the absence of bicarbonate, elongation of leaves and/or petioles in *R. crispus* was not stimulated by light. The specific photosynthetic activity was lower than in *R. maritimus* (0.13 vs. 0.19 μmol O_2 $m^{-2}s^{-1}$, Table 4), but this was compensated by a larger leaf area (139 ± 22 cm^2 in *R. crispus* and 46 ± 8 cm^2 in *R. maritimus).* Initial biomass of all plant parts was maintained to within 90% of the starting mass, and mortality was 0% after 20 and 30 days.

Addition of inorganic carbon (10 mM $NaHCO_3$) to the water significantly stimulated

Table 4 Effect of light intensity and of addition of sodium bicarbonate to the water on underwater photosynthetic activity of *R. crispus* and *R. maritimus* after 20 d of total submergence (means of 6 replicates ± SE; age of the plants, inorganic carbon concentration and treatments denotation as in Table 3)

	dark	light	light + C
		(μmol O_2 $m^{-2}s^{-1}$)*	
R. crispus	0	0.13 ± 0.02	0.20 ± 0.01
R. maritimus	0	0.19 ± 0.02	0.27 ± 0.01

*Calculated from the volume of gas released from submerged plants, assuming the released bubbles to contain 21% oxygen (M.B. Jackson, pers. comm.).

photosynthetic activity (Table 4) and elongation of the shoot in *R. maritimus* and of leaves in *R. crispus* (Table 3). With *R. maritimus* all plants survived a 30 days submergence treatment and growth in mass by roots and shoots was observed. Older shoot parts remained green, elongated, and new leaves were formed. In addition, a flower stalk with fast-growing adventitious roots on the nodes developed underwater and new, aerenchymatous roots were formed below-ground. Biomass production of the different plant parts was in all cases higher than the initial value (Table 3). In *R. crispus,* the impact of added bicarbonate on biomass of the different plant parts was even greater than in *R. maritimus:* tap-root biomass was increased almost fourfold, biomass of lateral roots twofold and shoot biomass threefold after the experimental period (Table 3).

Contribution of photosynthetic oxygen to survival

Because growth of *R. crispus* and both growth and survival of *R. maritimus* plants were dependent on light and/or on the inorganic carbon content of the water (Table 3), underwater photosynthesis must have played an important role in the responses performed by the plants. Not only was there a significant release of oxygen into the water (Table 4), the beneficial effect for the plant is that photosynthetically-derived oxygen can diffuse downward, in this way recovering or maintaining aerobiosis of the root system.

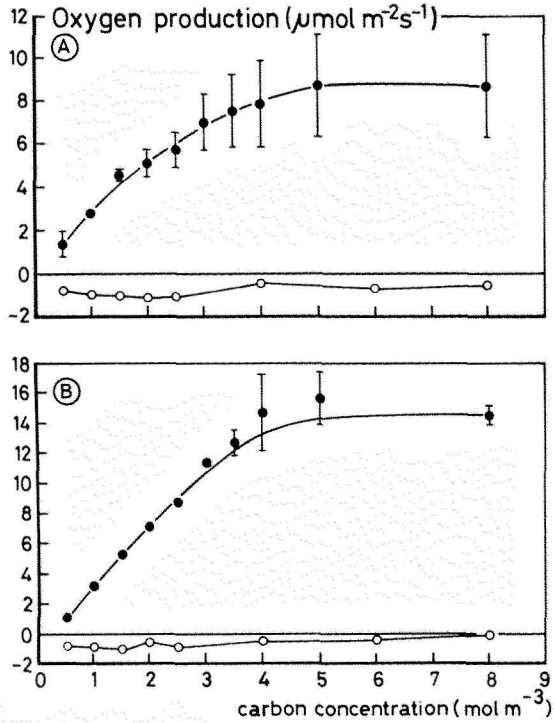

Figure 2 Photosynthetic oxygen production of leaf discs of *R. crispus* **(a)** and *R. maritimus* **(b)** at different bicarbonate (○) and CO_2 (●) concentrations in buffer solution (means of 2 replicates ± SE; measurements in 0.05 M phosphate buffer solution at pH 8.0 (bicarbonate) or pH 4.95 (CO_2); dark respiration of the discs at maximal HCO_3^- concentration: *R. crispus* 8.7 ± 0.6, *R. maritimus* 7.1 ± 1.3 μmol $m^{-2}s^{-1}$)

Both *R. crispus* and *R. maritimus* exclusively used CO_2 as inorganic carbon source (Fig. 2), although the specific photosynthetic rate of *R. maritimus* appeared to be almost twice as high than that of *R. crispus* (15 and 8.5 μmol $m^{-2}s^{-1}$ respectively, Fig. 2).

Figure 3 shows the effect of light irradiation and the addition of inorganic carbon (as CO_2) on the contribution of photosynthetically-derived oxygen to root respiration ('internal aeration') in hydroculture. When 5 mM CO_2 and enough light were supplied to the submerged shoots, more than 50% of the total root oxygen consumption could be attributed to photosynthetically-derived oxygen (Fig. 3). At a lower CO_2-concentration (0.5 mM), more than 10% of root respiration was due to shoot-derived oxygen. At both high and low

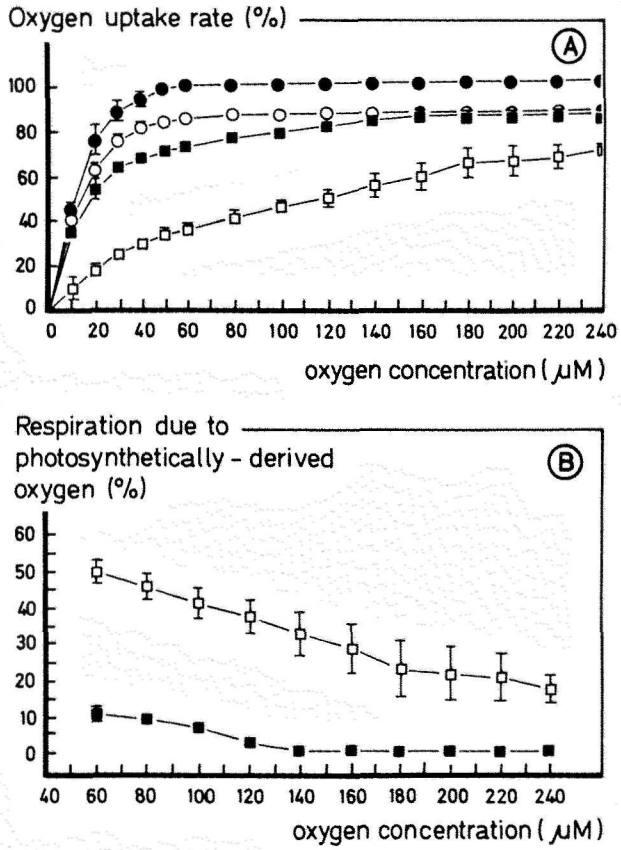

Figure 3 **(a)** Effect of light irradiation and of different CO_2-concentrations in the water on oxygen depletion of root systems of totally submerged *R. maritimus* plants, which were anaerobically pretreated for one week. Shoots in the dark (○), in light and 0.5 mM CO_2 (■), in light and 5 mM CO_2 (□), and of decapitated plants (●). Oxygen uptake rate as a percentage of maximal root respiration rate of decapitated plants at 240 µM O_2 in the root environment (= 100%, which was 83.2 ± 19.0 µmol O_2 g^{-1}DW h^{-1}); means of 3 replicates ± SE; age of the plants 4 weeks plus 1 week anaerobiosis; shoot compartment filled with 3.4 * 10^{-3} m^3 0.05 M NaH_2PO_4 (pH 4.95); different CO_2-concentrations achieved by the addition of $NaHCO_3$; light intensity at the leaves 500-800 µmol $m^{-2}s^{-1}$,
(b) Percentage contribution of shoot-derived photosynthetic oxygen to root respiration at different oxygen concentrations in the root environment at 0.5 (■) and 5 (□) mM CO_2 in the water (means of 3 replicates ± SE; data derived from Fig. 3a).

CO_2-concentration, the amount of photosynthetically-derived oxygen used by the roots increased with decreasing solution oxygen concentration (Fig. 3b). Since the oxygen uptake rate from the root environment of decapitated plants is higher than that of plants with

their shoots in the dark (Fig. 3a), oxygen can either be taken up from the water by the shoot, transported downwards and used for root respiration, or decapitation leads to a stimulation of root respiration.

DISCUSSION

The results show that the flood-tolerance of the *Rumex* species is strongly dependent on the inundation level used in the experiments. Upon soil flooding, the flood-intolerant *R. thyrsiflorus* cannot cope with sustained periods of root anaerobiosis, while the flood-tolerant *R. crispus* and *R. maritimus* can (Table 1). These responses are in accordance with earlier results (Laan *et al.* 1989b), and can be explained on the basis of a differential capability to develop root aerenchyma (Laan *et al.* 1989a). In *R. maritimus* and *R. crispus,* the development of an aerenchyma system leads to the avoidance of anoxia in the root system by internal longitudinal oxygen transport (Laan *et al.* 1990).

Upon total submergence of the plants, responses are directed towards survival and the maintenance of biomass. Although a strong reduction in growth rate was recorded for all species, the 'flood-tolerant' *R. maritimus* suffered, especially on submergence, because shoot elongation did not result in protrusion of leaves above the water surface (Table 1 and Fig. 1). This seems surprising, since *R. maritimus* occupies the lowest altitudinal level in the river foreland ecosystem, being confronted there with irregular periods of prolonged submergence during its growing season (Blom 1990).

The responses of *R. crispus* and *R. maritimus* to total submergence, however, clearly reflect their different life-histories. Apparently, *R. maritimus* relies predominantly on a fast shoot growth and on the allocation of biomass towards lateral roots rather than to the tap-root. Consequently, this requires a high supply of oxygen and sugars. Therefore shoot elongation, which can restore a free diffusion pathway between the air and the root system is of vital importance for this species (Fig. 1).

When the water surface is not reached, the plant may use underwater photosynthesis to satisfy its need for oxygen and sugars (Table 4, Fig. 3, Gaynard & Armstrong 1987; Setter *et al.* 1987). Survival of *R. maritimus* plants resulted only when the inorganic carbon supply is sufficient to sustain a high photosynthetic activity (Tables 3 & 4); the concomitant transport of photosynthetically-derived oxygen (Fig. 3) enables the plant to maintain a high root respiration rate.

It should be noted that the submerged *Rumex* species predominantly use dissolved CO_2 as inorganic carbon source (Fig. 2); hence, in the greenhouse experiments, where carbon was supplied as bicarbonate, an increase in photosynthesis can be most probably explained from the fact that the absolute amount of dissolved CO_2 was constantly higher in the bicarbonate-enriched containers compared to the containers poor in carbon content. Addition of 10 mM bicarbonate to tap-water results in a final bicarbonate concentration of 11.2 mM and a pH of 8.35; according to Stumm & Morgan (1970), this gives rise to a free, dissolved CO_2-concentration of 16 µM. Next to this, significant amounts of free CO_2 can be supplied via soil respiration. Moreover, this high content of bicarbonate leads to a relatively high buffering capacity of the solution, and thus a pH drift is unlikely to take place. Alternatively, the plants may have generated CO_2 by a local acidification of the water around the leaves, as was shown for *Potamogeton* and *Elodea* (Prins *et al.* 1982).

The impact of the carbon content of the water on survival could be mainly observed in *R. maritimus*. Since photosynthetic activity appeared to be very high (Fig. 2, Table 4), plus the fact that survival of this species seems to depend mainly on oxygen availability, a depletion of carbon and thus, the death of the plants, may easily occur.

Comparable results were obtained by Setter *et al.* (1987) with totally submerged rice plants: by flushing 3% CO_2 through the water, growth rate was increased five-fold. Gaynard & Armstrong (1987) found similar results with *Eriophorum angustifolium* and concluded that a significant improval of root aeration and rhizosphere oxygenation could be attributed to photosynthetically-derived oxygen upon total submergence. They also showed that

even at very low light intensities (15 μmol $m^{-2}s^{-1}$) oxygen supply to the root apex was improved. Thus, if in *R. maritimus* the shoot does not reach the water surface, increased light capture due to shoot elongation can be useful, if enough bicarbonate is present in the water. Indeed, bicarbonate concentration in the river water can be high (2.5 mM, Rijkswaterstaat 1968).

It is interesting that under totally submerged conditions, virtually no shoot elongation took place in the dark, while in light, leaves and petioles of especially *R. maritimus* showed significant increase in length (Table 3). A combination of both photosynthetic and photomorphogenetic aspects may explain these differences in elongation response. Since ethylene accumulation, in combination with the gibberellin GA_3 and/or with auxins, is of major importance in the elongation response of several plant species, including *Rumex* (Musgrave *et al.* 1972; Walters & Osborne 1979; Horton & Samarakoon 1982; Métraux & Kende 1983; Ridge 1987; Voesenek & Blom 1989), it seems plausible that responses are, at least partly, acting via ethylene.

Light enables the plant to perform photosynthesis (Table 3), and thus an improved oxygen and/or sugar status throughout the plant is likely to occur. Since hypoxia stimulates ethylene synthesis (Jackson 1982, 1985; Jackson *et al.* 1984; Raskin & Kende 1984), photosynthesis leading to hypoxic conditions (3-5% O_2) in the plant would promote shoot elongation. In addition, an improved sugar status might lead to a continuation of osmotic water uptake and thus to an increased cell expansion.

In combination with this, light itself may stimulate shoot elongation, either via a decrease in photon flux density, as was shown for *Hippuris vulgaris* (Spence *et al.* 1987) or via an altered light regime, as was shown for *Sorghum vulgare* (Craker *et al.* 1973); light, especially in the blue and far red regions of the spectrum, induced ethylene production.

When neither the water surface is reached, nor enough carbon plus oxygen can be generated by photosynthesis, reserves are soon exhausted and *R. maritimus* plants die

(Table 3, Fig. 1). For *R. crispus* photosynthesis is probably less important for survival (Tables 2 & 3), but the amount of respirable sugars stored in the tap-root may be an important factor determining survival chances of the plants. It remains however, difficult to correlate the amount of reserve carbohydrates and their consumption rate by the roots with survival chances of the plants upon total submergence. In the experiments conducted, the occurrence of underwater photosynthesis does not allow a certain interpretation of this correlation and, in addition, older *R. crispus* plants perform differently from younger plants, because respiratory demand of the root system strongly decreases with age, while the tap-root gets increasingly important as a sink for growth (Laan *et al.* 1990; P. Laan, unpublished results).

Rumex crispus, a perennial species, tends to allocate energy to the tap-root, while *R. maritimus,* the annual species, does not. The ratio tap-root : total biomass at the start of the submergence treatments was 0.03 for the young plants of *R. maritimus* and 0.07 for *R. crispus* (Table 3). Thus, with regard to the availability of reserves, survival chances of *R. crispus* are higher than those of *R. maritimus.*

In conclusion, for the annual species *R. maritimus* flood-tolerance seems to depend on oxygen availability, which is characterized by the inability to slow down metabolism. Upon total submergence, all responses are directed towards gaining oxygen, either by shoot elongation and protrusion of leaves above the water surface (Fig. 1), or by underwater photosynthesis (Table 4, Fig. 2). For the long-living perennial species *R. crispus,* oxygen availability is less important, and there may be a tendency to slow down metabolism upon submergence, contributing to the preservation of starch reserves in the tap-root and consequently to the capacity for regrowth on de-submergence (Tables 2 & 3). This difference in life-history stresses the importance of sugar supply: with *R. maritimus* relying more on the generation of sugars from photosynthesis, and *R. crispus,* relying more on the use of stored carbohydrates.

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GROWTH AND DEVELOPMENT OF *RUMEX* ROOTS AS AFFECTED BY HYPOXIC AND ANOXIC CONDITIONS

SUMMARY

Growth characteristics of three *Rumex* species, occurring in the river foreland ecosystem and differing in their flood-tolerance, were determined under different solution oxygen concentrations. The flood-tolerant *R. maritimus* and *R. crispus* developed a large number of new, aerenchymatous roots within a short period under low solution oxygen concentrations and biomass production was not affected. In the flood-intolerant *R. thyrsiflorus* however, only few, slow-growing new roots were developed and biomass production was significantly reduced at solution oxygen concentrations below 2%. These different responses could be partly explained from a differential aerenchyma formation in new roots of the flood-tolerant species, as aerenchyma can relieve the oxygen stress of the root systems via internal aeration. The fast development of new roots of the flood-tolerant *R. maritimus* and *R. crispus* after the onset of anaerobiosis in the root environment coincided with the reduction or cessation of growth of the primary roots. Notwithstanding the cessation of growth however, primary roots of both species were able to recover growth after renewed aerobic conditions following a 13-day anaerobic period. On the contrary, roots of *R. thyrsiflorus* ceased growing very soon after the onset of anaerobiosis and had all died after 10 days. The balance between growth characteristics of the primary and the newly formed root system are discussed and related to the differential tolerance of the *Rumex* species to transient flooding.

INTRODUCTION

Under hypoxic conditions root growth soon ceases, since the oxygen concentration near the root apex rapidly reaches values below the Critical Oxygen Pressure for Extension growth (COPE)(Armstrong & Webb 1985). In addition, ion uptake is strongly reduced (John *et al.* 1974; Drew & Sisworo 1979; Trought & Drew 1980b; Datta 1985; Thomson *et al.* 1989) and these processes result in the reduction of plant growth rate (Drew & Sisworo 1979; Trought & Drew 1980a; Buwalda *et al.* 1988). Plants can overcome these adverse effects by the formation of new, aerenchymatous roots which provide a basis to restore oxygen supply to the root tip (Armstrong 1979; Laan *et al.* 1989a, 1990).

Rumex species occur in the river foreland ecosystem, there infrequently being confronted with transient flooding (Blom *et al.* 1990). In their response to flooding, *Rumex* species develop new roots to different extents (Laan *et al.* 1989a,b) and with the

flood-tolerant ones the development of aerenchyma in these new roots enables the plant to perform internal oxygen diffusion which relieves the oxygen deficiency of the root system (Laan *et al.* 1990). Thus, the extension rate of the new root system will ultimately determine the flood-tolerance of the species. However, since the development of new roots takes some time, the plant must rely on the primary root system during the initial period after transient flooding.

In the present study we investigated growth responses of three *Rumex* species to different solution oxygen concentrations. Next to this, the growth balance between the primary and newly formed root system upon alternating aerobic and anaerobic hydroculture conditions was studied to investigate whether the turnover from old to new root system was directed towards a maintainance of high oxygen concentrations throughout, which may explain the differential tolerance of the species to transient flooding.

MATERIALS AND METHODS

Plant growth at different solution oxygen concentrations

Seeds of *R. maritimus* L., *R. crispus* L. and *R. thyrsiflorus* Fingerh. were collected from plants in the river forelands near Nijmegen (The Netherlands) and sown in trays containing black polyethylene grains (Stamylan LD, DSM, The Netherlands). The trays were filled with ¼ x Hoagland's solution (Hoagland & Arnon 1950) until total submergence of the grains. After germination (16 h light (Philips TL-33) at 60 µmol $m^{-2}s^{-1}$, 25°C and 8 h dark, 15°C), the germination trays were transferred to a temperature room (16 h fluorescent light at 200 µmol $m^{-2}s^{-1}$, 8 h dark, temperature 25°C, R.H. 70%) and allowed to grow for another 1-2 weeks. Then the seedlings were carefully transferred to black polyethylene pots (volume 1.5 l, 4 plants per pot), filled with nutrient solution, which was kept air-saturated by bubbling with air. Each plant was sealed into the air-tight lid with modelling-clay to prevent air contact. Nutrient solution was maintained at the original level every two days and replaced once a week.

After one week the root system was completely covered with active charcoal powder to be able to distinguish old from newly formed root material and, the plants were grown under different solution oxygen concentrations, using gasblenders (HI-TEC model E55N3). After 10 days root length of the longest new root and total length of newly formed root material were measured. Dry weights of newly formed root material and of the remainder of the plants were determined after 24 h at 70°C.

Growth of roots upon alternating aerobic and anaerobic conditions

Plant growth. Plants were grown as described above in a modified ¼ x Hoagland's solution containing 1.0 mM KNO_3, 1.0 mM $NaNO_3$ and 0.5 mM KH_2PO_4 instead of 1.5 mM KNO_3 and 0.75 mM $NH_4H_2PO_4$ in a temperature room (16 h light (Sylvania F36W-GRO, 150 µmol $m^{-2}s^{-1}$), 8 h dark; temperature 25°C, R.H. 70%).

Experimental setup. A two to three weeks old plant was carefully transferred to a cuvette consisting of two glass plates (width 0.2, height 0.6 m), which were fixed together with 4 mm thick glass strips. The space between the plates was filled with glass beads (diameter 4 mm) to fix the roots and, the cuvette was closed at the top with glass rods to minimize air contact. The system was placed at an angle of ca. 20° to allow the roots to grow at the frontside. The bulk nutrient solution (10 l container), which could be kept aerobic or anaerobic by bubbling either air or nitrogen gas, was circulated through the cuvette by flowing from bottom to top with an aquarium pump (Eheim 1016, 1 l h^{-1}). The whole system was blackened to prevent growth of algae. Measurements were started after ca. one week, when the longest roots had reached a length of 7-10 cm.

Growth measurements. Root growth was measured by marking the positions of the apices twice a day on a transparent sheet which was fixed at the frontside of the cuvette. Growth of newly formed roots was recorded as soon as they were detectable and, their originating time was calculated afterwards by assuming the growth rate of these laterals to be constant until then. Roots were registered dead when growth rate had reduced to zero and the apex had turned brownish or black. Average growth rates were calculated by measuring the distances between the markings on the sheet of 5 to 7 representative roots.

RESULTS

Plant growth at different solution oxygen concentrations

At low solution oxygen concentrations root growth of all species was restricted, but especially *R. thyrsiflorus* suffered from hypoxic or anoxic conditions in the root environment. A sharp decrease in root length was recorded below solution oxygen concentrations of 2%, which was especially true for *R. thyrsiflorus,* where primary roots ceased growing and died away, and only few, slow-growing new roots developed (Table 1). Also plant biomass was lowest at solution oxygen pressures of 0 and 1% in *R. thyrsiflorus,* but hardly affected in both *R. maritimus* and *R. crispus.* With these two latter species, highest biomass production was found at 2% O_2.

Within the experimental period new root material was formed. At higher solution oxygen concentrations new root material was mainly produced because of a continued growth of the already existing roots, but with decreasing oxygen concentrations new roots were

Table 1 Effect of different solution oxygen concentrations on root growth and biomass production (growth of 5 weeks old plants for 10 days; means of 32 replicates ± SE; data as a percentage of controls = 21% (v:v) O_2)

Species/ $[O_2]$ (% v:v)	Root length		Biomass	
	longest new root	total length of newly formed laterals	newly formed laterals	plant
R. thyrsiflorus				
21	19.8±1.2 cm=100%	31.4±2.2 cm=100%	3.1±0.3 mg=100%	0.17±0.01 g=100%
10	154 ± 6	187 ± 17	180 ± 19	133 ± 14
4	120 ± 5	125 ± 12	93 ± 11	90 ± 5
2	86 ± 6	114 ± 9	90 ± 12	101 ± 9
1	9 ± 2	19 ± 4	17 ± 5	56 ± 7
0	12 ± 1	31 ± 4	26 ± 5	56 ± 7
R. crispus				
21	29.4±1.8 cm=100%	35.5±2.8 cm=100%	3.4±0.4 mg=100%	0.19±0.02 g=100%
10	102 ± 6	174 ± 12	200 ± 26	151 ± 11
4	91 ± 4	202 ± 14	179 ± 15	109 ± 10
2	84 ± 3	381 ± 2	421 ± 35	164 ± 9
1	43 ± 3	208 ± 16	291 ± 29	128 ± 10
0	37 ± 3	139 ± 11	171 ± 18	89 ± 5
R. maritimus				
21	13.2±0.8 cm=100%	19.9±1.2 cm=100%	1.3±0.3 mg=100%	0.06±0.01 g=100%
10	100 ± 3	109 ± 7	169 ± 46	134 ± 10
4	90 ± 4	117 ± 21	154 ± 46	116 ± 11
2	95 ± 5	114 ± 13	192 ± 15	140 ± 11
1	57 ± 2	136 ± 20	269 ± 77	113 ± 11
0	58 ± 3	98 ± 19	169 ± 54	96 ± 8

developed. This was especially true for *R. crispus* and to lesser extent also for *R. maritimus,* but, as mentioned before, virtually no new roots were formed in *R. thyrsiflorus* . These differences are reflected in the total length of new root material formed after 10 days (Table 1): largest increases were observed at low solution oxygen concentrations in *R. crispus* and *R. maritimus,* wheras a sharp decrease was recorded in *R. thyrsiflorus* below 2% O_2.

New roots, formed in response to low solution oxygen concentrations could be easily distinguished from the primary ones as they were unbranched and, due to the formation of

aerenchyma (Laan *et al.* 1989a), their diameter was much higher (Table 2). Since the more efficient response of the flood-tolerant *R. maritimus* and *R. crispus* was mainly due to the development of large numbers of these newly formed lateral roots, they are expected to have a higher growth rate than the primary ones. The balance between the turnover from primary to new roots with respect to growth was therefore further investigated.

Table 2 Root diameter (in mm) of primary and newly formed lateral roots of *Rumex* species measured 2 cm behind the root apex (means of 15-20 replicates ± SD)

Species	primary laterals	newly formed laterals
R. thyrsiflorus	0.38 ± 0.00	0.62 ± 0.04
R. crispus	0.65 ± 0.09	0.72 ± 0.02
R. maritimus	0.43 ± 0.06	0.84 ± 0.04

Growth of roots upon alternating aerobic and anaerobic conditions

With all *Rumex* species under study, anaerobic conditions in the rooting medium ultimately resulted in a cessation of growth of the primary roots (Fig. 1). Growth rates of primary roots of *R. thyrsiflorus* and *R. crispus* were reduced rapidly after the onset of anaerobic root conditions and most roots had ceased growth completely after 48 and 56-80 h respectively (Fig. 1). In *R. maritimus,* however, growth reduction could not be observed until 90-100 h after the onset of anaerobiosis and complete growth reduction of the primary lateral roots was reached after ca. 170 h. Growth of these primary laterals could not be recovered throughout the anaerobic treatment and, in *R. thyrsiflorus* most roots had died after 240 h (Fig. 1).

With all species, new roots were developed after growth of the primary roots was reduced, but their time of origin strongly differed. In case of *R. thyrsiflorus* new, slow-growing roots could not be observed until the primary roots had ceased growth for 65 h; in *R. crispus* new root formation coincided with complete growth reduction of most of the

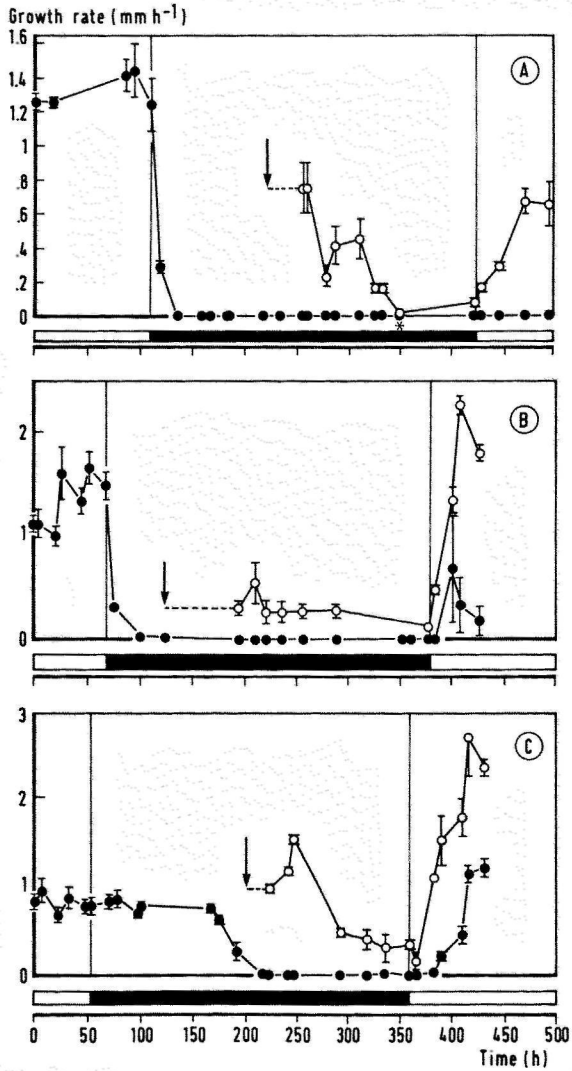

Figure 1 Growth rates of primary (●) and newly formed (○) lateral roots of *Rumex* species upon alternating aerobic (▭) and anaerobic (▬) conditions: **(a)** *R. thyrsiflorus,* **(b)** *R. crispus* and **(c)** *R. maritimus* (means of 5-7 replicates ± SE; arrow indicates origin of new root formation, * indicates death of primary lateral roots; age of the plants at the start of the experiment 3-4 weeks)

primary roots and their initial growth rate was also rather low (Fig. 1). With *R. maritimus* however, new roots began to grow out when primary roots showed a reduction in growth rate, but had not ceased growth completely yet, i.e. ca. 14 h before growth reduction to zero new roots were already growing out (Fig. 1). Next to this, these roots showed higher initial growth rates than the primary ones.

With increasing length, new roots also showed growth reduction under these experimental conditions and 230 h after the onset of anaerobiosis, the short newly formed roots of *R. thyrsiflorus* had stopped growing completely. Growth reduction due to increasing length of *R. maritimus* roots coincided with the formation of new roots, again with high initial growth rate.

Upon renewed aerobic conditions, growth rates of the new roots were strongly increased (Fig. 1) to 2-2.5 mm h^{-1} in *R. crispus* and *R. maritimus,* which was much higher than their initial growth rates and also than growth rates of primary roots under aerobic conditions. Although growth of new *R. thyrsiflorus* roots was increased upon renewed aereobiosis, the rate was still rather low (0.6 mm h^{-1}, Fig. 1) and did not exceed values obtained from the primary laterals under aerobic conditions. Growth of primary roots of both *R. maritimus* and *R. crispus* recovered after renewed aerobic conditions, but as primary roots of *R. thyrsiflorus* were dead, they could not restore growth.

DISCUSSION

Although anaerobic conditions in the rooting medium finally resulted in a complete cessation of growth of primary laterals of all species, it took much longer to fully reduce growth rates of *R. maritimus* roots compared to the other two species (Fig. 1). Also, maximal root length reached under low solution oxygen concentrations was least affected in *R. maritimus* (Table 1). This clearly reflects the differential root porosities and concomitant ability to use internal aeration for satisfying root oxygen demand (cf. Armstrong 1979; Laan *et al.* 1989a, 1990). Since porosity of the primary roots of *R.*

maritimus is twice as high as that of both *R. thyrsiflorus* and *R. crispus* (Laan *et al.* 1989a), oxygen diffusion via internal aeration will be higher in *R. maritimus* compared to the other two species. Given the fact that oxygen loss and respiratory characteristics of all three species is comparable (Laan *et al.* 1989b, P. Laan unpubl. data), apical oxygen pressures will sooner reach values below the critical oxygen pressure for extension growth (COPE; Armstrong & Webb 1985; Laan *et al.* 1989b) so that primary roots of *R. crispus* and *R. thyrsiflorus* will cease growth at shorter length.

The differences in internal aeration may also account for the differential growth rates of the newly formed roots of the species. Due to the formation of aerenchyma, new roots of *R. crispus* and *R. maritimus* have much higher porosity than the primary roots (Laan *et al.* 1989a), hereby providing a potential for high growth rates to occur. Nevertheless, initial growth rates of new roots of these two species under anaerobic conditions were rather low compared to the primary roots under aerobic conditions (Fig. 1). Apparently, anaerobic conditions in the cuvette were so severe that even growth of the highly porous new lateral roots was inhibited. It is most likely to assume the high circulation rate of the nutrient solution to withdraw oxygen from the roots, resulting in rather low apical oxygen concentrations. High growth rates of the new roots of *R. maritimus* and *R. crispus* were reached after renewed aerobic conditions (Fig. 1). Due to a relatively low porosity of new roots of *R. thyrsiflorus,* renewed aerobic conditions did not lead to high growth rates.

Next to the higher growth rate per root, also the total number of new roots formed after the onset of hypoxic or anoxic conditions was much higher in *R. crispus* and *R. maritimus* than in *R. thyrsiflorus* (Table 1). The high rate of new root formation may counteract the reduced growth of the primary root system and may explain the fact that biomass production under low solution oxygen concentrations was hardly affected in *R. crispus* and *R. maritimus* as it was in *R. thyrsiflorus* (Table 1).

An important phenomenon, which may distinguish between the tolerant and the intolerant *Rumex* species under situations of transient flooding, can be found in the regrowth capacity of the primary roots. Roots of both *R. maritimus* and *R. crispus* were able to recover growth after a 13-day anaerobic period, while those of *R. thyrsiflorus* died after 240 h (Fig. 1). Obviously, in *R. maritimus* this is most likely due to the high porosity of the roots (Laan *et al.* 1989a), which enables internal oxygen diffusion to take place continuously (Laan *et al.* 1990). The differential response of *R. crispus* and *R. thyrsiflorus* is remarkable, since the primary roots of these species both contain 7-8% porosity (Laan *et al.* 1989a). In spite of this similarity however, young plants of *R. crispus* appeared to be able to satisfy root oxygen demand via internal aeration at low oxygen concentrations, while root oxygen demand of *R. thyrsiflorus* cannot be satisfied via this process (Laan *et al.* 1990). Therefore, *R. thyrsiflorus* has to rely mainly on metabolic adaptations to cope with anaerobiosis; indeed a very high ADH activity in combination with the onset of a 'Pasteur effect' was observed (Chapter 9, this thesis), but the constant need of large amounts of respirable sugars finally may have resulted in the death of the roots.

Since new root formation in both *R. crispus* and *R. maritimus* seemed to be somehow related to the functioning of the old root system, the most intriguing question arising from these experiments is: what mechanism triggers new root formation following anaerobiosis? In *R. crispus* new roots started growing out immediately after most of the primary roots had ceased growth and in *R. maritimus* outgrowth of new roots had started even before the complete growth cessation of primary roots (14 h, Fig. 1). Obviously, no efficient turnover from old to new roots was found in *R. thyrsiflorus,* as it took almost 3 days for the first roots to originate. It may be speculated that the outgrowth of new roots is a function of growth reduction or cessation of the major part of the primary roots, for in earlier experiments with *R. maritimus* we never observed an outgrowth of new, aerenchymatous roots until growth of the primary ones had reduced severely.

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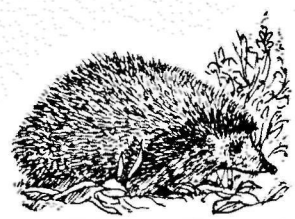
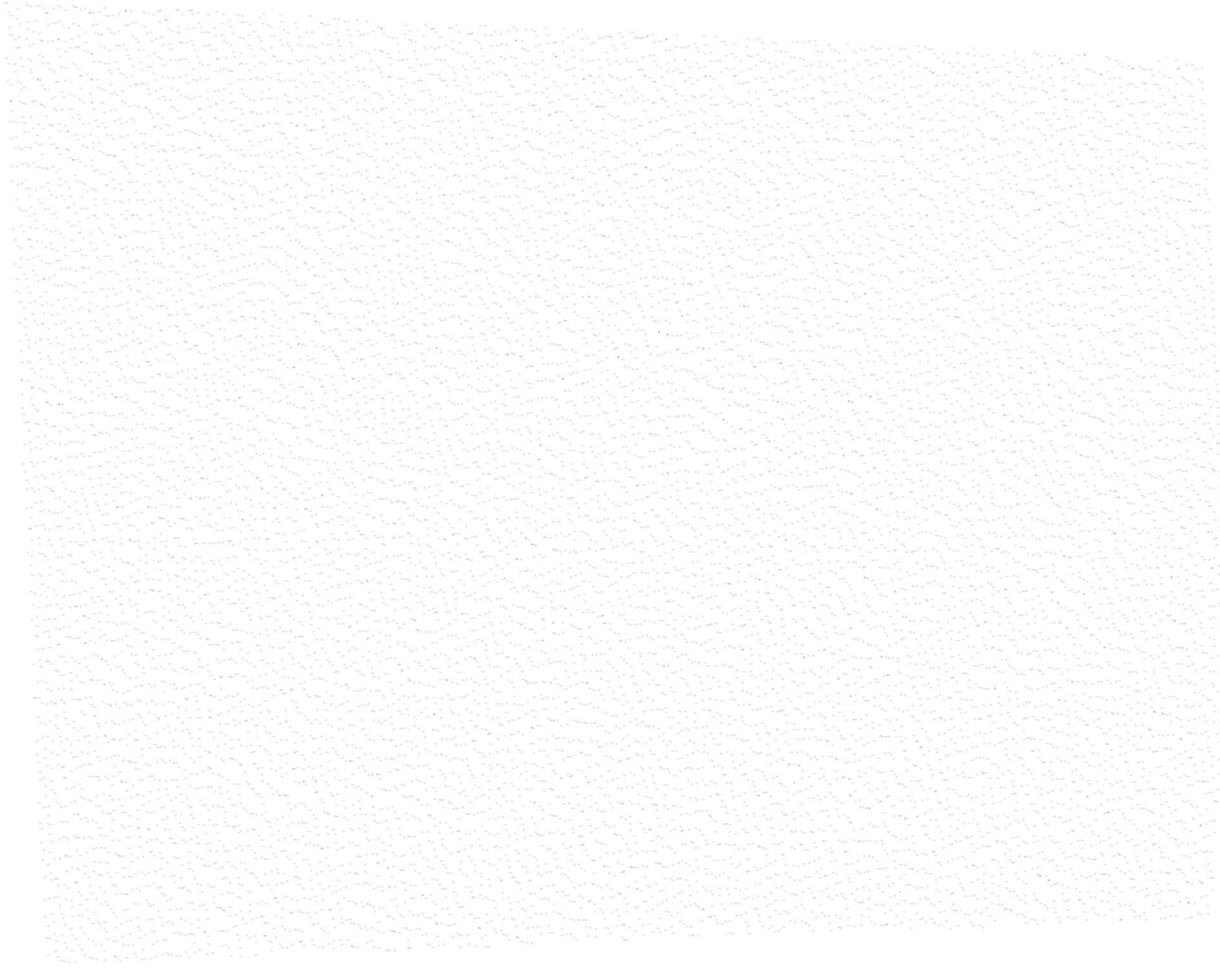
CHAPTER 8 The relative importance of anaerobiosis and high iron levels in flood-tolerance of *Rumex* species

with A. Smolders & C.W.P.M. Blom





THE RELATIVE IMPORTANCE OF ANAEROBIOSIS AND HIGH IRON LEVELS IN FLOOD-TOLERANCE OF *RUMEX* SPECIES

SUMMARY

The combined effects of flooding and high iron levels on growth and on the occurrence of iron toxicity symptoms were investigated in three *Rumex* species, differing in flood-tolerance.

In a hydroculture experiment the plants were subjected to different $FeCl_2$-concentrations and anaerobiosis. At solution iron concentrations exceeding 750 µM, the growth rate of especially the flood-intolerant *R. thyrsiflorus* was sharply decreased and mainly the root system was affected. It is concluded from these experiments that under hydroculture conditions the primary adverse effects on growth and development are restricted to the root system. Differences between the investigated species could be most likely explained from differences in root porosity and are thus closely related to a differential internal oxygen supply to the root systems.

In a greenhouse experiment soil-flooding was combined with the addition of different ferrous iron concentrations to the soil solution. Flooding in combination with the addition of 5 mM ferrous iron did not result in a significant decrease in biomass production of any of the investigated *Rumex* species, although several types of iron toxicity were perceived. Especially at high iron levels significant amounts of 'bronzing' spots on the leaves of all species could be observed; next to this, petiole toxicity symptoms, finally resulting in a bending down of the petioles was most clearly observed in the flood-intolerant *R. thyrsiflorus*. Since biomass production was hardly affected, it is concluded that high iron levels in the soil solution are of minor importance in the different flood-tolerance of the *Rumex* species.

INTRODUCTION

In waterlogged soils the available iron concentration is highly increased by the reduction of insoluble Fe(III)-oxides to Fe^{2+} (Ponnamperuma 1972, 1984; Yoshida & Tadana 1978); as a consequence, high internal iron concentrations in plant tissues may occur and reduce plant yield (Kuraev 1966; Fageria *et al.* 1987). In flood-tolerant plants, aerenchyma formation provides an efficient means to perform radial oxygen loss (ROL), which can help prevent high internal iron levels in the shoot by reoxidation and concomitant precipitation of iron in the rhizosphere (Chen *et al.* 1980; Ottow *et al.* 1982; Benckiser *et al.* 1984; Laan *et al.* 1989b). Nevertheless, high iron levels and symptoms of iron toxicity are not restricted to flood-intolerant plants, but, on the contrary, are almost exclusively

described for flood-tolerant species, including rice (Armstrong & Boatman 1967; Howeler 1973; Yoshida & Tadano 1978; Laan *et al.* 1989b).

The amount of iron accumulating in the plant is supposed to be the result of the oxidizing activity of the roots on the one hand, and of the transpiration rate of the shoot plus the iron concentration in the soil solution on the other (Jones 1971; Bienfait 1989; Laan *et al.* 1989b). In addition, the nutrient status of the plant can play a role in preventing iron toxicity: a low nutrient status may result in increased root exudation and concomitant rhizoflora activity, and thus in a high 'soil oxygen demand'. This can ultimately lead to the breakdown of iron excluding mechanisms (dissolution of iron precipitates), followed by an uncontrolled iron influx (Ottow *et al.* 1982; Benckiser *et al.* 1984). Therefore, iron toxicity is most likely to occur in flood-intolerant plants and in flood-tolerant plants growing on iron-rich, but nutrient-poor soils.

Although iron toxicity symptoms may occur in both shoot and root system, they are most frequently described for the shoot. A wellknown example is the socalled 'bronzing disease' in rice plants, characterized by the development of reddish brown spots on the lower (older) leaves (Ponnamperuma *et al.* 1955; Tanaka *et al.* 1966; Yoshida & Tadano 1978). Iron toxicity in the roots in most cases is characterized by root blackening and root flaccidity (Kuraev 1966; Tadano 1975; Wheeler *et al.* 1985). According to Kuraev (1966) the inhibition of root growth is initially caused by high Fe^{2+}-concentrations in the rooting medium. In many hydroculture studies, however, it is to be doubted whether root injury is caused by high Fe^{2+}-levels, rather than by sulphide toxicity. Experiments conducted on hydroculture in most cases are carried out with $FeSO_4$ (Wheeler *et al.* 1985; Van Diggelen 1988) and as sulphide is highly toxic to roots (Tanaka *et al.* 1968; Yoshida & Tadano 1978), root injury due to sulphide toxicity is more likely to occur than to iron toxicity in these cases.

In this study we investigated the influence of the combination of anaerobiosis and high iron concentrations on the growth of three *Rumex* species, differing in oxidizing

activity and concomitant flood-tolerance (Laan *et al.* 1989a,b). In a greenhouse experiment the occurrence and the nature of shoot iron toxicity symptoms were studied for plants grown in flooded, iron enriched soils. In a hydroculture experiment, growth rates and both root and shoot iron toxicity symptoms of the three *Rumex* species in an anaerobic medium containing different $Fe(Cl)_2$-concentrations were studied.

MATERIALS AND METHODS

Plant growth

Seeds of *Rumex thyrsiflorus* Fingerh., *R. crispus* L. and *R. maritimus* L. were collected from natural populations in the river area near Nijmegen (The Netherlands). They were sown in trays containing black polyethylene grains (Stamylan LD, DSM, The Netherlands), which were totally submerged with a 1/4 strength Hoagland's solution (Hoagland & Arnon 1950). The trays were covered with a glass plate and placed in a germination cell for 1-2 weeks (25°C (day), 15°C (night); 16 h fluorescent light (Philips TL 33) at 60 µmol $m^{-2}s^{-1}$, 8 h dark). After germination the young plants were allowed to grow for 2-3 weeks in a growth room (temperature 25°C, RH 70%; 16 h fluorescent light at 200 µmol $m^{-2}s^{-1}$, 8 h dark).

Hydroculture experiments

Plant growth. Plants, grown as described above, were carefully transplanted to containers (13 x 10^{-3} m^3), filled with 1/4 full strength modified Hoagland's solution (see Laan *et al.* 1990) and aerated. They were allowed to grow aerobically for a week (growth room conditions), after which the solution was replaced by a stagnant, 0.05% (w:v) agar solution. Plants were grown in this medium until they had reached a mean total fresh weight of 5 g and were then used for the experiments. At that moment the larger part of the primary lateral roots had died away and new laterals had developed.

Experimental design. Plants were weighed and planted in air-tight plastic pots filled with a stagnant anaerobic 0.1% (w:v) agar in 1/4 strength iron free Hoagland's solution. Six Fe^{2+}-concentrations, containing of 8 replicates each, were obtained by adding different amounts of an anaerobic $FeCl_2$-stock solution to the pots. Every second day the solutions were changed to prevent a strong decrease of the free iron concentration by oxidation. Before changing the solution, the Fe^{2+}-concentration was checked using the 'bipyridyl method' (according to Laan *et al.* 1989b). The maximum decrease after two days did not exceed 25% of the original concentration.

Plants were harvested when the mean total fresh weight was doubled. Fresh weights of the total plant, shoot and lateral roots and the maximum length of the laterals were measured. Relative growth rates of separate plants were determined from the total fresh weights of the plants at the start and at the end of the experiment. The number of leaves with visible iron toxicity symptoms ("bronzing spots" which always appeared on the oldest leaves) were counted. All plant parts were dried separately (24 h, 70°C) and dry weights were recorded. Leaves were divided into two classes, (i) leaves without iron toxicity symptoms and (ii) leaves with iron toxicity symptoms, and iron concentrations were determined (see 'Leaf iron concentration').

Greenhouse experiments

Experimental design. Plants, pregrown as described above were transferred to vertical PVC tubes (diameter 12 cm, height 40 cm) which were filled with a 1:1 (v:v) clay/sand mixture (pH (H_2O) 6.9; weight percentage organic matter 5.4) which was enriched with some homogenously distributed plant material. The plants were allowed to grow for 4 weeks in a greenhouse (temperature 19C, RH 70%, 16 h light, consisting of daylight filtered by greenhouse glass (120-1000 µmol $m^{-2}s^{-1}$) and supplementary light (high pressure sodium lamp, Osram Vialox), when light intensity decreased to values lower than 200 µmol $m^{-2}s^{-1}$; 8 h dark).

After another four weeks the tubes were flooded either with tap-water or with tap-water enriched with different $FeCl_2$-concentrations. Prior to the flooding treatment, air in the free spaces of the soil was replaced by nitrogen gas by flowing nitrogen gas via glass pipettes (diameter 3 mm; length 35 cm), positioned down to the bottom of the soil column, for 25 min. Then, an anaerobic $FeCl_2$-solution was led through the pipettes into the soil columns. In this way the oxygen-free soil columns were gradually saturated with the anaerobic iron solution, while oxidation was prevented and thus assuring a more or less equal distribution of Fe(II) throughout the soil columns. The waterlevel was kept at three centimeters above the soil by adding tap water twice a day. The 'free' iron concentration was determined several times during the flooding treatment, using the 'bipyridyl-method' (according to Laan *et al.* 1989b).

After a three weeks treatment, the plants were harvested by carefully pushing out the soil core including the root system. Soil oxidation depth, indicated by a brownish colour, and clearly distinguishable from the reduced black soil, was recorded and maximum root penetration was determined during removal of the soil. Shoots were cut off and fresh weight was determined. The root system was carefully washed out and fresh weight of the tap-root and lateral roots were determined separately. Dry weight of the several plant parts was determined after 24 h (70C) and, leaf iron content was determined as described below.

Leaf iron concentration. Total iron content of the leaves was determined on 50-100 mg dry leaf material ashed at 650C, dissolved in 5 ml 5% (v:v) HNO_3 and 0.5 ml 6 N HCl and analyzed on an inductive-coupled-plasma emission spectrophotometer (ICP).

RESULTS

Hydroculture experiment

Growth of *Rumex* species in anaerobic hydroculture was inhibited by Fe^{2+}-ions (Fig. 1); this effect was strongest in *R. thyrsiflorus*. Typical symptoms of iron toxicity were root flaccidity and root blackening at the higher iron concentrations. In the shoot, the oldest leaves showed brown necrotic spots. These 'bronzing spots' are considered to be typical for leaf iron toxicity (Foy *et al.* 1978; Wheeler *et al.* 1985; Yoshida & Tadano 1978). The number of injured leaves increased with increasing ferrous iron levels in the nutrient solution. At intermediate concentrations (750 µM) the total number of leaves affected was

ca. 20% in all species, but in contrast with the small effect of ferrous iron on RGR and root growth, injury was strongest in *R. maritimus* at high ferrous iron concentrations (40% of the total number of leaves at 1500 µM Fe, Table 1).

In old leaves, those with visible injury contained iron concentrations twice that of

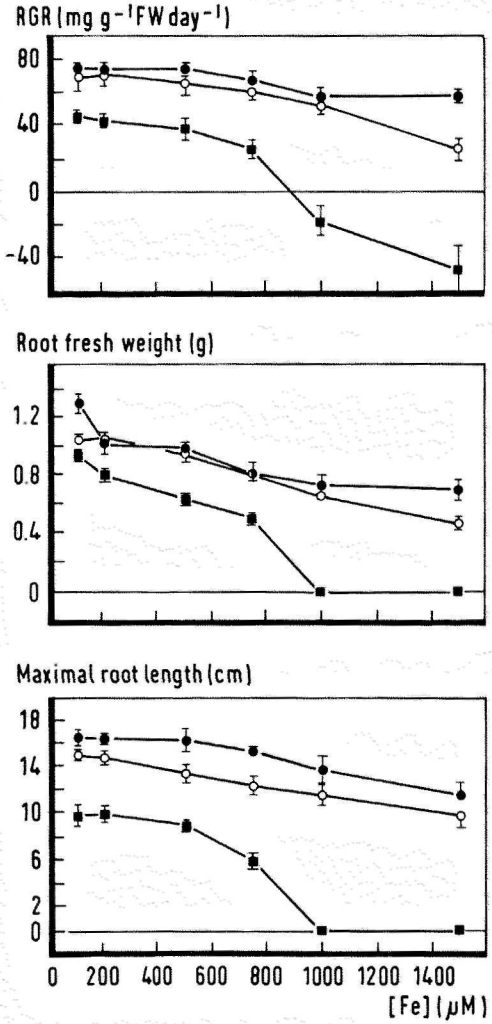


Figure 1 Combined effect of anaerobiosis and different concentrations of $FeCl_2$ on growth rate **(top)**, root fresh weight **(middle)** and maximal root length **(bottom)** of *Rumex* species after growth for 10 *(R. maritimus* and *R. crispus)* or 15 *(R. thyrsiflorus)* days in hydroculture; (○) *R. maritimus,* (●) *R. crispus,* (■) *R. thyrsiflorus* (means of 4 replicates ± SE)

leaves without symptoms, irrespective of species and concentration used (Table 1). However, although RGR and root growth of *R. thyrsiflorus* were most severely affected at high iron levels, iron concentration of the leaves was lower than of the other two species.

Table 1 Effect of anaerobiosis plus different $FeCl_2$ concentrations in the nutrient solution on visible iron toxicity symptoms and on leaf iron concentration of *Rumex* species (means of 8 (% injury) or 4 replicates ± SE; data after growth for 10 *(R. crispus* and *R. maritimus)* or 15 *(R. thyrsiflorus)* days in hydroculture)

Species/ $[Fe^{2+}]$	Number of leaves with visible injury due to iron toxicity	Leaf iron concentration	
		old leaves with injury	leaves without injury
	(% of total)[1]	(μmol g^{-1} DW)	
R. thyrsiflorus			
100 μM Fe	0	-	6 ± 1
750 μM Fe	18	26 ± 5	13 ± 3
1500 μM Fe	27	31 ± 4	18 ± 4
R. crispus			
100 μM Fe	0	-	4 ± 1
750 μM Fe	22	32 ± 8	12 ± 3
1500 μM Fe	18	42 ± 5	21 ± 4
R. maritimus			
100 μM Fe	0	-	4 ± 1
750 μM Fe	21	24 ± 3	14 ± 3
1500 μM Fe	40	45 ± 6	27 ± 5

[1]SE maximal 7%

Greenhouse experiment

When the clay soil column was pushed out of the tube, an oxidized zone (brownish) could be distinguished from a reduced zone (black) and the majority of the roots was found in this oxidized zone (Laan *et al.* 1989b). According to what we found earlier (Laan *et al.* 1989b), the depth of oxidation differed between the species and was correlated with oxidizing activity of the roots due to aerenchyma formation (ca. 30 cm in *R. maritimus,* ca. 15 cm in *R. crispus* and only 7 cm in *R. thyrsiflorus,* Table 2).

Table 2 Biomass production, iron-oxidation depth and ferrous iron concentration in the soil solution after 3 weeks of flooding of *Rumex* species, which were subjected to different iron levels under soil-flooded conditions (means of 8 replicates ± SE; growth in a clay:sand (1:1 v/v) soil, and ferrous iron concentration determined in the reduced soil layer; age of the plants at the start of the treatment 6-7 weeks; control = soil-flooded, 1 and 5 mM Fe = soil-flooded plus addition of $FeCl_2$; actual $[Fe^{2+}]$ in soil solution after 18 d in control, 1 mM and 5 mM Fe-treatment: 0.24 ± 0.05, 0.33 ± 0.09 and 1.15 ± 0.18 mM, respectively)

Species/ Treatment	Biomass production (g)		Oxidation depth (cm)
	Shoot	Roots	
R. thyrsiflorus			
control	2.2 ± 0.2	0.63 ± 0.04	8.2 ± 0.3
1 mM Fe	2.1 ± 0.2	0.60 ± 0.04	6.4 ± 0.4
5 mM Fe	2.3 ± 0.2	0.54 ± 0.05	5.8 ± 0.2
R. crispus			
control	2.6 ± 0.1	2.4 ± 0.2	15.8 ± 0.5
1 mM Fe	2.4 ± 0.1	2.3 ± 0.1	15.8 ± 0.5
5 mM Fe	2.5 ± 0.2	2.6 ± 0.4	13.9 ± 0.7
R. maritimus			
control	3.8 ± 0.6	1.9 ± 0.4	31.5 ± 0.8
1 mM Fe	4.4 ± 0.3	2.3 ± 0.4	28.6 ± 1.1
5 mM Fe	4.9 ± 0.4	2.7 ± 0.4	25.0 ± 0.9

Since soil sink activity for oxygen increases with increasing ferrous iron concentration, we expected the oxidation depth to be severely reduced upon addition of ferrous iron to the soil solution. Indeed, oxidation depth decreased, but to a much lesser extent than expected and no differences were observed between the species (reduction of 18% at 5 mM Fe, Table 2). This minor effect can be partly explained from the interaction between root oxidizing activity and 'free' iron concentration; this 'free' ferrous iron concentration had decreased significantly after 18 days of treatment, and could hardly be detected in the oxidized soil layer (<0.01 µM). In the reduced soil layer flooding itself gave rise to a $[Fe^{2+}]$ of ca. 0.24 mM (Table 2), but the extra addition of iron to a calculated concentration of 1 and 5 mM resulted in an actual concentration of 0.33 mM and 1.15 mM, respectively (Table 2). Moreover, even the highest ferrous iron concentration applied did

not affect biomass production significantly (Table 2) and typical root iron toxicity symptoms (root flaccidity and root blackening) could only be observed in the reduced soil layer.

The shoots showed typical forms of iron toxicity on both petioles and leaves, which was especially clear at higher iron concentrations (Table 3, Fig. 2). Necrotic spots ('bronzing spots') appeared on the leaves of all species under study (up to ca. 20% of the total number of leaves, Table 3). Next to this, just above the water surface different stages of petiole injury developed (Fig. 2). This type of injury was characterized by a die back of the cortical tissue, finally resulting in a bending down of the petioles (Fig. 2e). This bending was especially found with *R. thyrsiflorus*, to a much lesser extent in *R. crispus* and only with a few leaves of *R. maritimus* (Table 3). Wilting of leaves containing petiole toxicity could only be observed until the latest stages of this type of injury, i.e. after bending down. The injured parts of the petioles (Fig. 2) contained high amounts of iron, while the apical petiole parts beyond and the leaves had 2-5 times lower iron

Table 3 Visible injury of leaves and petioles of *Rumex* species, after 3 weeks of soil-flooding in the absence or presence of additional ferrous iron (data are means of 8 plants ± SE, and represent percentage of total number of leaves or petioles with injury)

	Injury on petioles (%)		Injury on leaves (%)
	weak	severe	
R. thyrsiflorus			
control	0	0	18 ± 3
1 mM Fe	0	0	12 ± 3
5 mM Fe	30 ± 7	18 ± 5	15 ± 3
R. crispus			
control	0	0	0
1 mM Fe	0	0	3 ± 2
5 mM Fe	24 ± 9	19 ± 7	13 ± 3
R. maritimus			
control	0	0	0
1 mM Fe	0	0	19 ± 3
5 mM Fe	2 ± 1	2 ± 1	11 ± 2

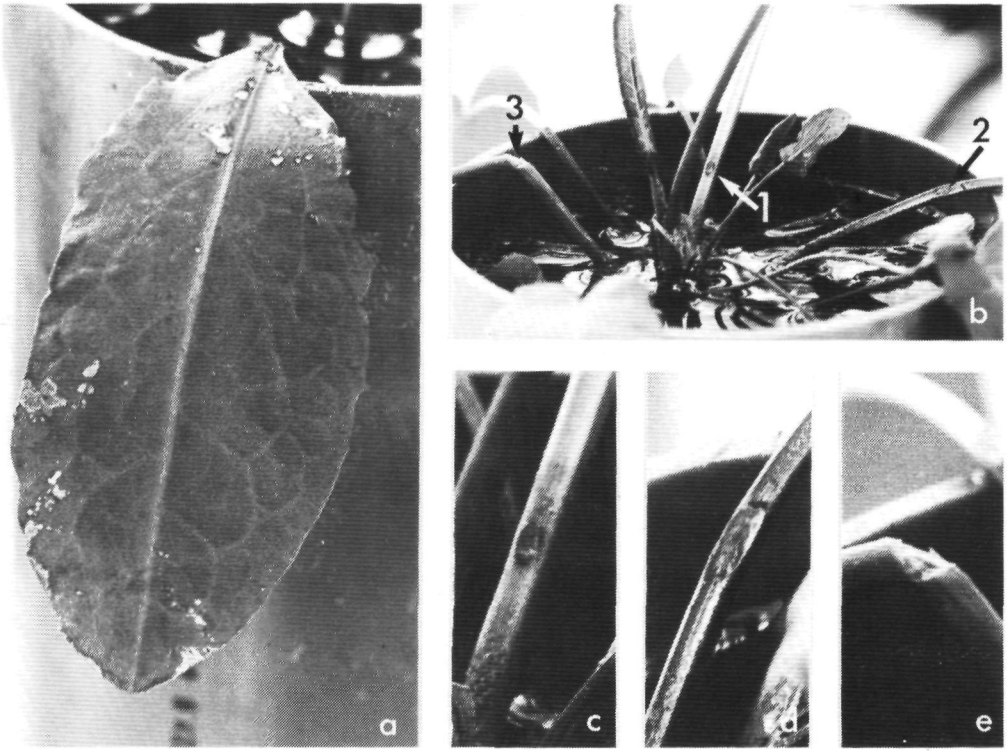


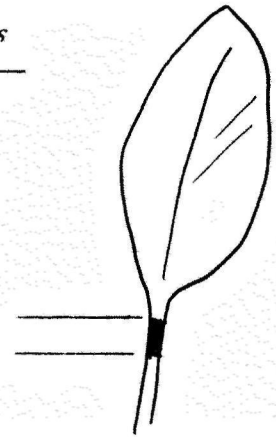
Figure 2 Appearance of different iron toxicity symptoms on leaves and petioles of *Rumex* species after a 3-week growth period in a flooded clay:sand soil, enriched with 5 mM $FeCl_2$. Leaf iron toxicity ('bronzing') in *R. crispus* **(a)** and several stadia of petiole iron toxicity in *R. crispus,* indicated with an arrow: overview **(b)** and magnifications of different stadia **(c-e)**; **(c)** stadium 1, **(d)** stadium 2, **(e)** stadium 3

contents (Table 4). On a dry weight basis, the injured petiole parts of *R. thyrsiflorus* contained significantly lower iron concentrations than those of *R. crispus* and *R. maritimus* (14 vs. ca. 25 µmol g^{-1}DW respectively, Table 4).

Typically, the appearence of petiole injury in no case agreed with the occurrence of leaf injury, i.e. 'bronzing spots'.

Table 4 Iron concentration of leaves and petioles of *Rumex* species with petiole injury (plants grown under soil-flooded conditions plus 5 mM $FeCl_2$; means of 3 replicates ± SE; data in brackets represent iron concentration of comparable leaves without petiole injury)

R. thyrsiflorus	*R. crispus*	*R. maritimus*
Iron concentration (μmol g^{-1}DW)		
6.1 ± 0.9 (13.9 ± 2.5)	8.4 ± 1.6 (14.4 ± 0.8)	6.8 ± 1.6 (18.7 ± 1.5)
3.0 ± 0.4	7.6 ± 0.7	12.3 ± 1.3
14.4 ± 0.3	24.1 ± 10.2	25.6 ± 3.5
4.6 ± 0.3	10.1 ± 1.1	19.1 ± 3.7



DISCUSSION

Hydroculture experiment

In the hydroculture experiment two factors have played a role in the adverse effects on growth of the plants, i.e. anaerobiosis and high ferrous iron concentrations. At low iron concentrations (100 μM) anaerobiosis led to a growth reduction, that was most severe in *R. thyrsiflorus* (Fig. 1). This is in accordance with earlier results (Laan *et al.* 1989a,b) and is mainly due to differences in root porosity of the species (Laan *et al.* 1989a). Since the lack of aerenchyma formation in *R. thyrsiflorus* roots leads to a poor internal aeration of the root apices (Laan *et al.* 1989b, 1990), this also explains the poor root development and the restricted root length (Fig. 1).

With increasing ferrous iron concentration in the nutrient solution, growth rates of all species under study was further reduced and this was accompanied by a corresponding decrease in root development. Again the effect was strongest in *R. thyrsiflorus* (Fig. 1). As already suggested by Kuraev (1966), the initial effect of anaerobiosis in combination

with high iron levels is the inhibition or cessation of root growth and development.

Contrasting with root responses, leaf iron concentration and the resulting degree of leaf injury was highest in *R. maritimus* (Table 1), which appeared to be least sensitive on the basis of growth rate (Fig. 1). The appearance of shoot iron toxicity symptoms is highly dependent on shoot iron concentration (Tanaka *et al.* 1966; Yoshida & Tadano 1978; Wheeler *et al.* 1985) and since iron uptake rate depends on water uptake (Jones 1971), this is most likely to occur in plants with a high transpiration rate and a well-developed root system, i.e. *R. maritimus*.

Greenhouse experiment

Under flooded conditions, all investigated *Rumex* species were able to oxidize the upper part of the soil (Table 2), resulting in very low 'free' iron concentrations (< 0.01 mM, Laan *et al.* 1989b). Thus, although high iron concentrations were recorded in the reduced soil layer (Table 2), the bulk of root mass was always found in the oxidized layer, containing low iron concentrations. As a result, no significant growth reduction of either roots or shoot could be observed (Table 2). The mere effect of the increasing iron concentration in the soil solution was a slight decrease in oxidation depth (Table 2).

In spite of the occurrence of this 'avoidance' strategy, however, clear symptoms of iron toxicity could be detected in the shoot parts of all *Rumex* species (Table 3, Fig. 2). Typical 'bronzing spots', as were observed in hydroculture could be detected in all species (Fig. 2a). The initial stage of this 'bronzing' damage appeared as small, brown necrotic spots either at the leave edges or near the leaf tip. With prolonged exposure to iron, this necrosis expanded over the leaf and, especially in *R. thyrsiflorus* covered large parts of the area of older leaves.

Interestingly, the iron concentration of leaves of *R. maritimus* appeared to be higher than that of the other two species (Table 4). Nevertheless, the percentage of leaves showing 'bronzing' damage did not differ between the *Rumex* species, either in the 1 or 5 mM treatment (Table 3). It remains therefore unknown whether *R. maritimus* has additional

mechanisms to avoid leaf injury in spite of the higher iron accumulation e.g. ferritin induction (Bienfait 1989).

Iron toxicity leading to 'petiole bending' (Fig. 2) was mainly observed a few centimeters above the water surface. Obviously, ferrous iron, transported via the xylem, will be oxidized when it comes into contact with oxygen. This oxidation leads to the formation of $O_2^{\bullet -}$ and subsequently to other oxygen radicals which destroy the cell membrane (Bienfait 1989). The process may take place both in the apoplast and in the cell, after uptake of Fe. The ferric iron will precipitate on the spot, indicating whether oxidation takes place. Of course, the amount of iron precipitated is a function of both the rate of iron transported (determined by the transpiration rate) and the diffusion rate of oxygen (determined by porosity). This is illustrated by the fact that *R. maritimus*, of which the petioles have a very low diffusional resistance to oxygen (Laan *et al.* 1990), showed the highest iron contents, also in the zone just below the water level (i.e. below the petiole bend), while *R. thyrsiflorus*, with petioles with high resistance contained relatively low iron concentrations in and below the bend (Table 4).

The leaves with bended petioles apparently avoided wilting symptoms until the latest stages of injury, which can be explained by assuming that the transport tissue of the petioles remained intact, in this way enabling a continued water and nutrient transport to the leaves. Remarkably, iron accumulation in petiole-injured leaves was prevented (Table 4). From a physiological point of view, petiole injury can have advantages over leaf injury, as a significant loss of photosynthetic capacity can be avoided for some time.

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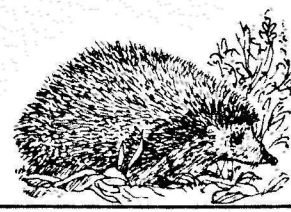
CHAPTER 9 Metabolic adaptation to root anaerobiosis: induction of anaerobic enzymes, occurrence of the 'Pasteur effect' and significance of nitrate in anaerobic responses of *Rumex* species

with J.M.A.M. Clement, E.J.W. Visser & C.W.P.M. Blom



METABOLIC ADAPTATION TO ROOT ANAEROBIOSIS: INDUCTION OF ANAEROBIC ENZYMES, OCCURRENCE OF THE 'PASTEUR EFFECT' AND SIGNIFICANCE OF NITRATE IN ANAEROBIC RESPONSES OF *RUMEX* SPECIES

SUMMARY

The occurrence of a 'Pasteur effect', the hypoxia-mediated induction of ADH, PDC and LDH and the possible importance of nitrate in anaerobic metabolism was studied in roots of three *Rumex* species to estimate the potential significance of metabolic adaptations in the flood-tolerance of the species. After a three days anaerobic pretreatment, the CO_2 production of roots of the flood-tolerant *R. crispus* had reduced to 78% of aerobic CO_2 production, indicating that a 'Pasteur effect' had developed with time in this species. After a five days pretreatment of young plants with different solution oxygen concentrations, the activity of the main 'anaerobic enzymes' appeared to be strongly increased below solution oxygen concentrations of 2% (v:v); ADH activity of the flood-intolerant *R. thyrsiflorus* had increased 40-fold, while that of the other two flood-tolerant species had increased less (9-and 17-fold in *R. maritimus* and *R. crispus,* respectively). PDC activity was also increased at low oxygen concentrations, but here the largest increase was observed with the flood-tolerant *R. maritimus,* while a significantly lower increase was obtained from *R. thyrsiflorus.* In these root extracts, up to 1.2 μmol g^{-1}FW of ethanol was found with both *R. crispus* and *R. maritimus* at low solution oxygen concentrations, but no ethanol could be detected in the roots of *R. thyrsiflorus.*

To study the possible role of NR in metabolic responses, plants were anaerobically pretreated for three days with or without nitrate and *in vitro* activities of ADH and NR were determined simultaneously. Increases in ADH activity upon anaerobiosis were comparably high as observed before, regardless of the pretreatment used. NR activity was threefold increased upon anaerobiosis in *R. crispus,* either in the presence or absence of nitrate in the nutrient solution. This suggests a function of NR in anaerobic metabolism for this species.

It is concluded that metabolic adaptations can be of significant importance for *R. crispus,* either to cope with short-term root anaerobiosis, or in certain root parts, where oxygen is unavailable; for *R. maritimus,* the significance of metabolic responses seems to be restricted to those root parts, that are insufficiently provided with oxygen. In the flood-intolerant *R. thyrsiflorus,* metabolic responses do not seem to be of a large importance in counteracting oxygen deficiency of the root system.

Abbreviations: PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; NR, nitrate reductase; BSA, bovine serum albumine; PVPP, polyvinyl polypyrrolidone; HEPES, N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid; DTT, dithiotreitol; MES, 2-N-morpholino ethane sulfonic acid; NAD(H), nicotionamide adenine dinucleotide; NED, N-(1-naphtyl)-ethylene diamine-dihydrochloride.

A well-known metabolic response to anaerobiosis is the acceleration of glycolysis- and fermentation-reactions ('Pasteur effect', Turner 1951; Davies 1980). This phenomenon can partly counteract the negative effects of hypoxia on energy supply of the roots and a relatively high ATP-production can be achieved for some time (Drew *et al.* 1985; Saglio *et al.* 1988). This process is normally accompanied by a rise in the amount of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Wignarajah & Greenway 1976; Laszlo & St. Lawrence 1983), the induction of which occurs within 2-4 hours (Sachs *et al.* 1980).

In addition, it has been suggested that under oxygen deficiency, nitrate, as an alternative electron acceptor, may withdraw part of the surplus of NADH-equivalents by reoxidation (Arnon 1937; Garcia-Novo & Crawford 1973; Lambers 1976; Lambers *et al.* 1978; Prioult & Guyot 1985; Reggiani *et al.* 1985a,b; Veen 1989), hereby realizing a continuation of the glycolytic reactions. It was shown for cucumber, tomato (Veen 1989) and for wheat (Prioult & Guyot 1985) that NR-activity was increased upon hypoxic conditions and suggested that nitrate, as alternative electron acceptor, contributed to the accelerated glycolytic rate.

The actual significance of nitrate in anaerobic metabolism however, is not quite clear. Trought & Drew (1981) explained the beneficial effect of nitrate as an alleviation of the negative effects of anaerobiosis on the mineral nutrition of the plant and from a metabolic point of view, there is contrasting evidence that nitrate results in an improved NADH-disposal, leading to higher ATP-levels (Lee 1978; Prioult & Guyot 1985; Bertani *et al.* 1987; Saglio *et al.* 1988).

In the present study we compared metabolic responses of the roots of three *Rumex* species towards anaerobiosis. The *Rumex* species differ in flood-tolerance (Laan *et al.* 1989a,b) and are confronted with transient flooding during their growing season (Blom *et al.* 1990). The most outstanding morphological adaptation of the tolerant species is the

formation of new, aerenchymatous roots (Laan *et al.* 1989a), which contribute significantly to total root mass within 4-5 days (Laan *et al.* 1989a; Chapter 7, this thesis). During the first period of anaerobiosis, metabolic adaptations may partly compensate the lack of oxygen in certain root parts of both tolerant and intolerant *Rumex* species. We investigated whether a 'Pasteur effect' was induced and determined the *in vitro* activity of PDC, ADH and LDH of roots from plants grown at different solution oxygen concentrations. Next to this, the importance of the addition of nitrate to the anaerobic nutrient solution on ADH- and NR-activity and on the production of ethanol was examined.

MATERIALS AND METHODS

Plant growth

Seeds of *Rumex maritimus* L., *R. crispus* L. and *R. thyrsiflorus* Fingerh. were collected from natural populations in the river ecosystem and germinated in a germination chamber (16 h light at 60 µmol $m^{-2}s^{-1}$, 8 h dark; temperature 20°C (day), 15°C (night)) on containers filled with black polyethylene grains (Stamylan LD, DSM, The Netherlands), which were submerged with a nutrient solution (KNO_3 1 mM, $Ca(NO_3)_2.4H_2O$ 1 mM, $NaNO_3$ 0.75 mM, KH_2PO_4 0.5 mM, $MgSO_4.7H_2O$ 0.25 mM; FeEDTA 0.25 mM, KCl 12.5 µM, H_3BO_3 6.3 µM, $MnSO_4.H_2O$ 0.5 µM, $ZnSO_4.7H_2O$ 0.5 µM, $CuSO_4.5H_2O$ 0.1 µM, H_2MoO_4 0.1 µM; pH 5.5). After germination the plants were grown for 1-2 weeks on nutrient solution, aerated with air using a porous aeration stone, in closely sealed black polyethylene pots (1.5 l). These plants were either subjected to different oxygen concentrations for 5 days (gas-blenders HI-TEC model E55N3) and then used for the determination of ADH-, PDC- and LDH-activity, or grown for another 3-4 weeks on an aerated nutrient solution and then used for CO_2-production measurements.

Determination of CO_2-production

CO_2 produced by whole root systems was determined in a modification of a previously described system (Laan *et al.* 1990). This system consists of a thermostatted measuring cuvette with a net volume of 180 ml; a polarographic oxygen electrode, according to Kimmich & Kreutzer (1969) was placed into the vessel wall. At the opposite side a CO_2 electrode (Orion, model 95-02) was mounted into the vessel wall. Optimal mixing of the nutrient solution was achieved by stirring with a motor-driven set of paddle-boards, together with the positioning of three vertical baffles at the inner side of the vessel. The oxygen concentration was registered with an oxygen micro-sensor (Diamond Electro-Tech, Ann Arbor, USA), the production of CO_2 with a millivolt-meter (Metrohm-654 pH/mV meter). CO_2-production rates were calculated from the change in voltage, using a calibration curve.

With roots from aerobically grown plants, the depletion of oxygen and the simultaneous production of CO_2 was recorded, starting with a fully aerated nutrient solution down to 0 µM O_2 in the cuvette; then CO_2-production was determined for another 30 min in the

absence of oxygen. With roots from plants, which were anaerobically pretreated for 3 days, CO_2 production was first followed under anaerobic conditions for 1 h, then the nutrient solution in the cuvette was fully aerated and aerobic CO_2-production was registered.

Enzyme activity

Four batches of plants, each batch consisting of eight plants, were harvested after five days. Young lateral root material was homogenized in an ice-cold mortar with 1-2 ml extraction buffer (50 mM HEPES-buffer (pH 7.5) plus 5 mM DTT), 5% (w:w) PVPP and ca. 100 mg quartz-sand. The homogenate was centrifuged (20 min at 45,000 g, 0-4°C; Sorvall RC-5) and the supernatant used for determination of enzyme activities and soluble protein content. Protein concentration was determined in duplicate with the Bio-Rad protein assay according to Appenroth & Augsten (1987), using BSA, fraction V, as standard.

Determination of ADH-, PDC- and LDH-activity. *In vitro* activity of ADH, PDC, LDH was determined according to Bergmeyer (1974) by recording the initial decrease (1-5 min) in extinction (340 nm, 25°C) with a spectrophotometer (LKB Novaspec 4049 and Shimadzu UV-120-02), using 3-ml cuvettes. In all cases, enzyme activities were proportionally correlated to the volume used. ADH. 2 ml 50 mM MES-buffer (pH 6.5), 300 µl BSA (0.5 mg (ml 0.1% (w:v) NaCl)$^{-1}$), 150 µl 2.2 mM NADH and 200 µl root extract were added together and after gently shaking, the reaction was started by addition of 200 µl 50 mM acetaldehyde. PDC. 2 ml 200 mM citrate buffer (pH 6.0), 300 µl root extract, 100 µl 2.2 mM NADH, 10 µl ADH (10 mg ml^{-1}; spec. act. 400 U (mg protein)$^{-1}$), 25 µl 100 mM KCN were added together and after gently shaking, the decrease in extinction due to aspecific activity was recorded (1-2 min). The reaction was started by addition of 20 µl 900 mM Na-pyruvate. Aspecific activity in no case exceeded 10% of the enzyme activity. Measurements were performed within 1 h after extraction, as activity decreased by 50% within one day. LDH. 2 ml 50 mM HEPES buffer (pH 7.0), 300 µl BSA (0.5 mg (ml 0.1% (w:v) NaCl)$^{-1}$), 150 µl 0.2 mM NADH and 200 µl root extract were added together and after gently shaking, the reaction was started by addition of 100 µl 23 mM Na-pyruvate.

Determination of ethanol. Ethanol was determined enzymatically (Bergmeyer 1974) in the supernatants of the root extracts, which were used for the determination of enzyme activity, by adding together in a 3-ml cuvette: 2.5 ml 100 mM phosphate buffer (pH 9.0), 100 µl semicarbazide (2.2 M in 2N NaOH, pH 6.4), 10 µl 300 mM glutathion, 200 µl 27.7 mM NAD and 100 µl sample solution. The cuvette was sealed with 'Parafilm' and the reaction was started by the addition of 50 µl ADH (spec. act. 400 U mg^{-1}protein). Maximal extinction at 340 nm was measured after 20-30 min on a Shimadzu (UV-120-02) photospectrometer.

Experimental design for the determination of ethanol and for the simultaneous registration of NR- and ADH-activity

Plants of 4-6 weeks old were aerobically and anaerobically pretreated for three days, either in the presence of 8 mM NO_3^- (+nitrate treatment) or without nitrate (-nitrate treatment) in the nutrient solution. In the -nitrate treatment, nitrate was replaced by K_2SO_4 and $CaCl_2$ to compensate for K^+ and Ca^{2+}. These plants were transferred to 100-ml Erlenmeyer flasks, filled with the same solution in which they had been pretreated. The nutrient solution was made anaerobic in advance by thorough flushing with nitrogen gas (2 h). The plants were sealed with a mixture of clay and plasticine, in which glass capillaries were mounted. In case of the anaerobic treatment, the solution was gently

flushed with nitrogen gas during the experiment. Preceding the experiment, it was checked whether this flushing affected the solution ethanol concentration by determining the recovery of different ethanol concentrations in solution after 3 h. It appeared that virtually no loss of ethanol took place throughout the experimental period. In the aerobic treatment, the solution was gently flushed with air.

1-ml samples were withdrawn from the solution every half hour with the use of the capillaries, kept on ice and used for the determination of ethanol, using the method described before. After 3 h the roots were separated from the shoots and used for the determination of ADH and NR activity. Enzyme extraction for the determination of ADH activity and the ADH assay itself were conducted as described above.

NR activity. *In vitro* NR activity was determined by grinding roots with an extraction buffer solution (0.1 M K_3PO_4 (pH 7.5), 1 mM Na_2EDTA, 5 mM DTT and 1% (w:v) BSA), 5% (w:v) quartz sand and 5% (w:v) PVPP. Homogenates (total volume 8 ml) were kept on ice in the dark, and then centrifuged (20 min, 25,000 g at 0-4°C, Sorvall RC-5). NR activity (modified after Robin (1979)) was determined in the supernatant by adding together: 500 µl K_3PO_4 buffer solution (pH 7.5), 100 µl 0.1 M KNO_3, 100 µl 1.4 mM NADH and 200 µl sample solution. After incubation for exactly 20 min (30°C), the reaction was stopped by adding 100 µl 1 M zinc acetate to the mixture. Then 1 ml sulphanilamide (10 g l^{-1} in 3 N HCl) and 1 ml NED (0.01% w:v) were added. After another 30 min the mixture was centrifuged (10 min, 8,000 g) and the extinction of the supernatant was measured in 3-ml cuvettes at 540 nm (LKB Novaspec). NR activity was determined by comparing the extinction of the samples with those of a calibration series, consisting of known amounts of $NaNO_2$, which were treated in the same way as the samples. With both samples and standards, the volume used was proportional to the extinction.

RESULTS

'Pasteur effect' and the induction of anaerobic enzymes

With all *Rumex* species under study, CO_2-production was more or less halved compared to aerobic CO_2-production after an anaerobic incubation period of 30 min (ratio 0.4-0.5, Table 1). After an anaerobic period of 3 days, anaerobic CO_2-production had increased in *R. crispus* (from a ratio of 0.42 to 0.78, Table 1), but was maintained at the same level in the other two species. Since the theoretically expected ratio between aerobic and anaerobic CO_2-production equals 0.33 (i.e. 6 moles CO_2 produced per mol glucose under aerobic vs. 2 moles under complete anaerobic conditions), the more than twofold increase compared to the value of 0.33 recorded in *R. crispus,* indicates that a 'Pasteur effect' was induced in this species, that develops with time. The lower CO_2-ratios, obtained from both *R. maritimus* and *R. thyrsiflorus,* suggest that a 'Pasteur effect' was not developed.

An acceleration of glycolytic rate ('Pasteur effect') is normally accompanied by the

Table 1 Ratios between CO_2-production under anaerobic and aerobic conditions of *Rumex* roots after an anaerobic pretreatment of 30 min and after 3 days anaerobisis (means of 3 or 4 replicates ± SE; mean aerobic CO_2-production after 3 d was: *R. maritimus* 27.9 ± 6.0, *R. crispus* 23.4 ± 2.2, *R. thyrsiflorus* 14.3 ± 1.1 µmol g^{-1}FW h^{-1}; age of the plants 7-8 weeks)

Species	CO_2-production (anaerobic)/CO_2-production (aerobic)	
	after 30 min anaerobiosis	after 3 days anaerobiosis
R. thyrsiflorus	0.54 ± 0.05	0.55 ± 0.02
R. crispus	0.42 ± 0.02	0.78 ± 0.13
R. maritimus	0.47 ± 0.04	0.43 ± 0.10

induction of enzymes of the anaerobic fermentation reactions. Indeed, a strong increase in ADH-activity could be observed below solution oxygen concentrations of 2% (Fig. 1): in *R. thyrsiflorus,* ADH-activity increased almost 40-fold upon anaerobiosis (from 125 at 21% O_2 to 4800 µmol $min^{-1}g^{-1}$protein at 0% O_2, Fig. 1); in *R. crispus* it was 17 times higher. In *R. maritimus,* there was a more gradual increase in ADH-activity with decreasing solution oxygen concentrations and a 9-fold increase was recorded (from 125 at 21% O_2 to 1100 µmol $min^{-1}g^{-1}$protein at 0% O_2, Fig. 1). Obviously, lactate fermentation was of minor importance in *R. thyrsiflorus* and *R. crispus,* as LDH-activity was very low compared to ADH-activity (Fig. 1); in *R. maritimus* however, a 5-10 times higher value than observed in the other two species was recorded (up to 100 µmol $min^{-1}g^{-1}$protein, Fig. 1) and lactate fermentation could be of some significance in this species.

PDC-activity was increased under low solution oxygen concentrations (Fig. 1), although the absolute activity was much lower than that of ADH. In contrast to the ADH-activities however, PDC-activity was highest in *R. maritimus* and a sharp increase below 2% O_2 was obtained, while a more gradual increase with decreasing solution oxygen concentration was found in *R. thyrsiflorus* (Fig. 1).

Ethanol could not be detected in the nutrient solution, but in the root extracts, used for the determination of enzyme activities, ethanol concentrations appeared to be

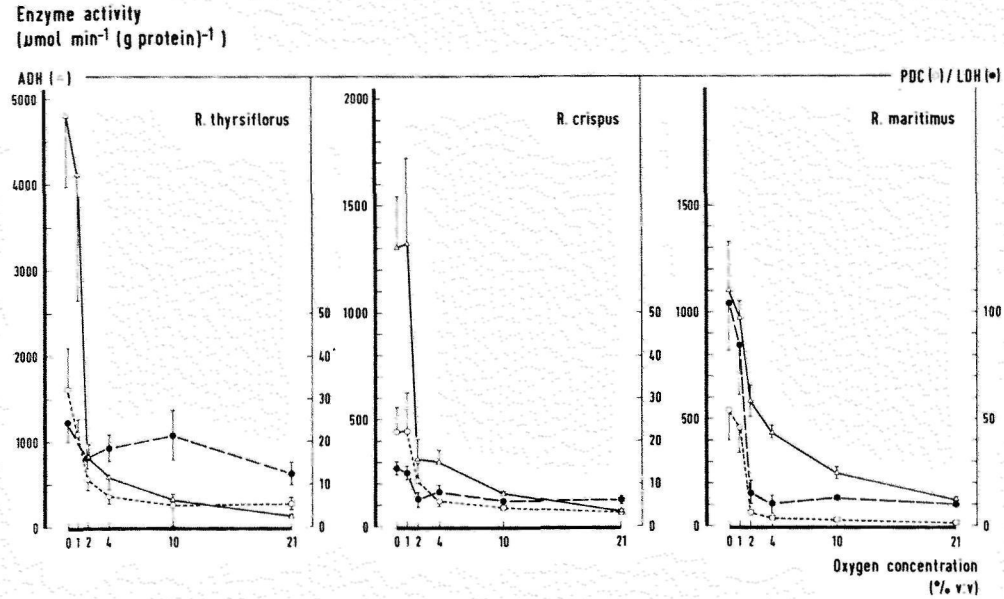


Figure 1 In vitro activities of alcohol dehydrogenase (△), pyruvate decarboxylase (○) and lactate dehydrogenase (●) of *Rumex* roots, which were subjected to different solution oxygen concentrations for 5 days (means of 5 replicates ± SE)

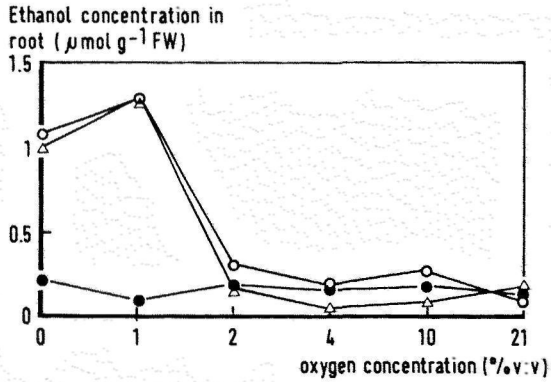


Figure 2 Ethanol concentration in root extracts of *R. thyrsiflorus* (●), *R. crispus* (○) and *R. maritimus* (△), subjected to different solution oxygen concentrations for 5 days

higher in both *R. crispus* and *R. maritimus* at low oxygen concentrations (up to 1.25 µmol g^{-1}FW, Fig. 2). Contrasting with the high ADH-activity found, virtually no ethanol could be detected in the root extracts of *R. thyrsiflorus* under anaerobic conditions (Fig. 2).

Nitrate in anaerobic metabolism

The possible importance of nitrate in metabolic responses of the *Rumex* species under anaerobic conditions was tested by determining NR-activity after an aerobic or an anaerobic pretreatment of the plants in the presence or absence of nitrate.

In the presence of nitrate, NR-activity was rather low under aerobic conditions in *R. thyrsiflorus* (0.93 μmol NO_2^- g^{-1}FW h^{-1}, Table 2) and anaerobiosis led to a small increase (+40%). A three-fold increase of NR-activity upon anaerobic conditions was observed in *R. crispus* (from 1.31 to 3.95 μmol NO_2^- g^{-1}FW h^{-1}, Table 2). NR-activity was very high already under aerobic conditions in *R. maritimus* (3.73 μmol NO_2^- g^{-1}FW h^{-1}, Table 2), but had slightly decreased upon anaerobiosis (to 78% of control value).

Plants of all three *Rumex* species under study, pretreated without nitrate, showed an increase in NR-activity upon anaerobiosis (Table 2). However, *R. maritimus* plants showed the opposite response compared to the +nitrate treatment: instead of a decrease, an 86%

Table 2 Effect of anaerobiosis and of the addition of nitrate on soluble protein content, nitrate reductase activity and alcohol dehydrogenase activity of roots of *Rumex* species (means of 5 replicates ± SE; plants pretreated for 3 days; numbers between brackets indicate percentage of control = aerobic conditions + or -nitrate; age of the plants 6 weeks)

	Nitrate reductase (μmol g^{-1}FW h^{-1})		Alcohol dehydrogenase (μmol g^{-1}FW min^{-1})		Protein content (mg g^{-1}FW)	
Species	+Nitrate	-Nitrate	+Nitrate	-Nitrate	+Nitrate	-Nitrate
R. thyrsiflorus						
aerobic	0.93 ± 0.22	0.28 ± 0.02	0.20 ± 0.07	0.27 ± 0.10	0.48 ± 0.09	0.36 ± 0.08
anaerobic	1.30 ± 0.15 **(140%)**	0.49 ± 0.14 **(175%)**	1.76 ± 0.35	0.63 ± 0.24	0.57 ± 0.08	0.18 ± 0.04
R. crispus						
aerobic	1.31 ± 0.14	0.19 ± 0.06	0.05 ± 0.01	0.06 ± 0.02	5.32 ± 0.44	4.40 ± 0.13
anaerobic	3.95 ± 0.69 **(302%)**	0.69 ± 0.08 **(363%)**	2.21 ± 0.44	2.02 ± 0.36	8.03 ± 0.42	5.80 ± 0.81
R. maritimus						
aerobic	3.73 ± 0.15	0.63 ± 0.10	0.51 ± 0.17	0.03 ± 0.01	1.72 ± 0.28	0.19 ± 0.04
anaerobic	2.92 ± 0.31 **(78%)**	1.17 ± 0.12 **(186%)**	3.52 ± 0.24	2.24 ± 0.12	2.06 ± 0.22	1.32 ± 0.10

increase in NR-activity was obtained under anaerobiosis in the absence of nitrate.

With all species, anaerobiosis either in the absence or presence of nitrate resulted in a higher soluble protein content. The only exception was *R. thyrsiflorus,* pretreated without nitrate; here protein content was halved upon anaerobiosis (Table 2), probably due to the poor vitality of the roots after 3 days. Withholding nitrate from the nutrient solution led to a decrease in soluble protein concentration. This effect was strongest in aerobically grown *R. maritimus* plants, where an almost 90% decrease could be observed (Table 2).

As with NR, ADH-activities of the root extracts are presented on a fresh weight basis. For our purpose, activities based on root fresh weight seem to give a more reliable measure than on a protein basis, since the plant actually benefits from absolute increases per unit root mass and not from the relative contribution of anaerobic enzymes to total protein content.

Comparable to the induction of NR-activity, ADH-activity was strongly increased upon anaerobiosis. Regardless of the pretreatment used, a strong increase in ADH-activity to more than 2 μmol g^{-1}FW min^{-1} was recorded in *R. crispus* (Table 2). A similar response was found in *R. thyrsiflorus,* but only in the presence of nitrate; in the -nitrate treatment, only a ca. twofold increase was recorded. *R. maritimus* showed the highest ADH activity upon anaerobiosis, especially in the presence of nitrate (3.52 μmol g^{-1}FW min^{-1}, Table 2).

In these experiments, in which we subjected whole plants to anaerobiosis for 3 hours, ethanol, diffused into the solution, could not be detected in any of the treatments.

DISCUSSION

Although higher CO_2-ratios than the theoretically expected value of 0.33 were obtained immediately after the onset of anaerobiosis with all species, only *R. crispus* showed a progressive increase of anaerobic CO_2-production with time (Table 1). The ratio between anaerobic and aerobic CO_2-production obtained (0.78) is in accordance with what is usually found for species that develop a 'Pasteur effect' (e.g. Girton 1979). Notwithstanding

the fact that the ratios obtained from *R. maritimus* and *R. thyrsiflorus* were higher than 0.33 immediately after the onset of anaerobiosis, the relativey low values obtained (0.4-0.5) plus the lack of a further increase of the anaerobic CO_2-production with time (Table 1), makes it unlikely to suggest a 'Pasteur effect' to be of significant importance in either of these species.

As an important characteristic of metabolic adaptation, the activity of the main key-enzymes of the anaerobic pathway were strongly increased at low solution oxygen concentrations; in particular, an increase of ADH- and PDC-activity was observed (Fig. 1). Such responses are in accordance with observations from many species, including flood-tolerant and intolerant ones (John & Greenway 1976; Wignarajah & Greenway 1976; Laszlo & St. Lawrence 1983). Although lactate fermentation in most cases forms a constitutive part of anaerobic metabolism of many plant species (Davies *et al.* 1974; Fagerstedt & Crawford 1986; Brändle 1990), it is less dominant than alcoholic fermentation (Davies 1986; ap Rees *et al.* 1987) and a significant increase in LDH-activity could only be detected in *R. maritimus* (Fig. 1).

As a relatively 'flood-intolerant' species, *R. thyrsiflorus* showed the highest ADH-activity on a protein basis, but this increase was not proportionally accompanied by a stimulation of PDC-activity (Fig. 1). Although absolute ADH-values were lower than in *R. thyrsiflorus*, PDC-activity was highest in the 'flood-tolerant' *R. maritimus*. The fact that PDC is thought to be more important in determining ethanol production than ADH (John & Greenway 1976), plus the observation that ethanol was only detected in root extracts of *R. crispus* and *R. maritimus* (Fig. 2), indicate that the PDC- rather than the ADH-activity determines the rate of glycolytic and fermentation reactions to continue. From this point of view, the more efficient metabolic responses are found among the 'flood-tolerant' species, as was proposed earlier (Phillips 1947; Bertani *et al.* 1980; Saglio *et al.* 1980; Ap Rees *et al.* 1987; Mendelssohn & McKee 1987).

The three-fold increase in NR-activity found for *R. crispus,* either in the presence or absence of added nitrate (Table 2), indicates that nitrate can be of significant importance in the metabolic responses of this species, and suggests a role for nitrate as alternative electron acceptor in anaerobic metabolism (cf. Garcia-Novo & Crawford 1973; Bertani *et al.* 1987). It is unlikely that a function for NR in anaerobic metabolism is restricted by a substrate shortage, as nitrate uptake rates remain positive, also after two weeks of anaerobiosis (P. Laan & E. Visser unpubl. results). To a lesser extent an increase in NR-activity was also observed in *R. thyrsiflorus,* so unlike the lack of the development of a clear 'Pasteur effect', NR may play a role in anaerobic metabolism of this species too. Since a decrease in the presence and an increase in the absence of nitrate was recorded in *R. maritimus* (Table 2), a function for NR in anaerobic metabolism of *R. maritimus* remains obscure.

Although young *R. maritimus* plants increased ADH- and PDC-activity (Fig. 1, Table 1) and produced ethanol (Fig. 2), the fact that no 'Pasteur effect' was developed (Table 1) points at the limited value of metabolic adaptations for the flood-tolerance of this species. *R. maritimus,* a wetland species, develops aerenchymatous roots, also under aerobic conditions (Laan *et al.* 1989a) and in fact a high porosity throughout enables the plant to satisfy root oxygen demand under hypoxic conditions for the larger part by internal longitudinal oxygen transport (Laan & Blom 1990; Laan *et al.* 1990). Therefore, the root systems of *R. maritimus* plants must have been aerated quite well during the experimental period, even under very low solution oxygen concentrations. Nevertheless, root parts with high respiratory activity, like the root apices and stelar root zones may remain in an anaerobic state, despite of the formation of an extended root aerenchyma (Armstrong 1989); this may easily lead to the induction of 'anaerobic enzymes' like ADH, as their induction will merely depend on the total number of cells, being in an anoxic state. Such phenomena may help to explain the apparently contrasting results between enzyme induction and the occurrence of a 'Pasteur effect'.

Notwithstanding the fact that aerobically grown *R. crispus* plants can use comparably high amounts of internally diffused oxygen for root respiration as *R. maritimus* under hypoxic conditions (Laan *et al.* 1990), both an increase in ADH- and PDC-activity and the development of a 'Pasteur effect' were observed (Tables 1 & 2, Fig. 1). Apparently, a larger part of the roots than in *R. maritimus* was insufficiently supplied with oxygen via internal aeration, resulting in the development of a 'Pasteur effect'. An extended state of root anaerobiosis is more likely to occur with *R. crispus* than with *R. maritimus* under severe anaerobic conditions, because *R. crispus* loses the ability of using internally transported oxygen for root respiration with age, mainly due to the increasing diffusive resistance of the tap-root for gas transport (Laan *et al.* 1990); in such situations, anaerobic root zones are likely to develop and metabolic adaptation may partly counteract for the adverse effects of oxygen deficiency in those root parts.

In the flood-intolerant *R. thyrsiflorus,* anaerobic responses seem to function quite inefficiently. Although a very high ADH activity was obtained (Fig. 1), neither the clear development of a 'Pasteur effect' (Table 2), nor the production of ethanol (Fig. 2) could be detected. As *R. thyrsiflorus* roots die in solution culture within one week (Chapter 7, this thesis), metabolic adaptations do not seem to be of significant importance in the resistance of *R. thyrsiflorus* roots to anaerobiosis. Since the lack of the ability to develop aerenchyma (Laan *et al.* 1989a) leads to a very low contribution of internally transported oxygen to root respiration (Laan *et al.* 1990), the root system will be devoid of oxygen within a short time; therefore, *R. thyrsiflorus* must rely on shallow rooting in combination with the formation of short roots to relieve the oxygen stress and thus to cope with anaerobiosis for some time (cf. Laan *et al.* 1989a,b).

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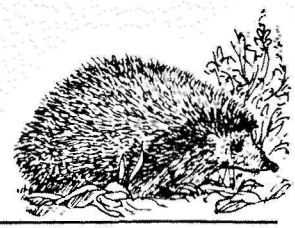
CHAPTER 10 General Discussion



GENERAL DISCUSSION

In this Chapter final conclusions will be summarized and integrated in an attempt to estimate the significance of the different phenomena which were studied for the flood-tolerance of the *Rumex* species. Since the responses strongly depend on the flooding level (Chapter 6), it is important to distinguish between flood-tolerance under partially and under totally submerged conditions. Partial flooding refers to both soil-flooding (waterlogging) of the plant, and to a state in which the plant is only protruding with a few leaf tips above the water surface; in most cases differences in flood-tolerance are reflected as differences in growth rate and biomass production. Under totally submerged conditions, the plant, including all shoot parts is completely submerged; differences in flood-tolerance emerge as differences in survival.

Aerenchyma formation in Rumex roots

Several characteristics of the *Rumex* aerenchyma type coincide with characteristics, that are suggested to be main prerequisites for flood-tolerance (Kawase 1981; Justin & Armstrong 1987). Roots of *Rumex* species show a 'cubic' cell packing, which can be observed in transverse section (Fig. 3, Chapter 2). This cell arrangement results in larger intercellular space volume and it was therefore suggested that plant species, having roots with cubic cell arrangement are more likely to develop flood-tolerance than those with hexagonal cell packing (Justin & Armstrong 1987). In non-flooded roots a relatively high porosity will result, which leads to a low diffusional impedance and thus to a better oxygen supply of the root cells; next to this, the cubic cell arrangement is proposed to be a configuration, that easily leads to aerenchyma formation and is therefore referred to as 'pre-aerenchymatous' (Justin & Armstrong 1987). Indeed, a 'pre-aerenchymatous' cell arrangement can be observed in aerobically grown *R. maritimus* roots (Fig. 3, Chapter 2).

In general, we cannot simply distinguish between tolerant and intolerant species by the fact whether they form aerenchyma or not. A much more realistic measure of the actual flood-tolerance may be given by the combination of root porosity and cell arrangement (Justin & Armstrong 1987), as this highlights the balance between oxygen supply and oxygen requirements in terms of diffusion and diffusional resistances. Figure 1 gives an overview of the porosity under drained conditions and the capability to increase porosity upon anaerobiosis. The three *Rumex* species, which were subject of most of this thesis, are included in the overview of 'non-wetland' and 'wetland' plant species. Most 'wetland' species are characterized either by a high root porosity under both drained and flooded conditions (constitutive aerenchyma formation) or, by the ability to increase porosities upon flooding (inductable or facultative aerenchyma formation). On the contrary, 'non-wetland' species have low porosities and are unable to increase porosity when flooded. All three *Rumex* species included in figure 1 show high root porosities and in fact, are incorporated within the 'intermediate' and 'wetland' species.

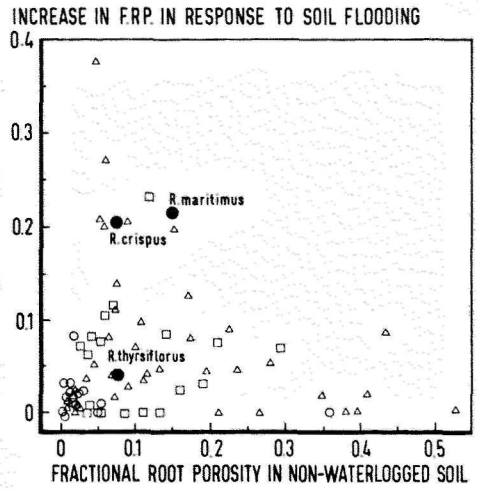


Figure 1 Increase in fractional root porosity (FRP) in response to soil flooding of a large number of 'wetland' (△), 'intermediate' (□) and 'non-wetland' (○) species (Redrawn from Justin & Armstrong 1987; data of *Rumex* species from Chapter 2)

Considering the suggestion that root porosity under drained conditions plus the ability to increase porosity upon anaerobiosis (Fig. 1) are main determinants for flood-tolerance, it appears that *Rumex* species have developed a unique combination of these characteristics and apparently have adapted efficiently to the fluctuating water levels, due to transient flooding: under drained conditions, they already have quite a porous root system (at least 7% porosity in the 'intolerant' *R. thyrsiflorus,* Chapter 2), while under flooded conditions, new roots are formed, containing the typical 'honeycomb' aerenchyma type in the tolerant species.

The schizogenously formed *Rumex* aerenchyma type contrasts on two points with lysigenously formed aerenchymas. In the latter ones, aerenchyma formation not only increases root porosity, but also reduces total cell number and thus the oxygen demand per unit volume (Williams & Barber 1961; Armstrong 1979; Jackson & Drew 1984), whereas in general an increase in total root volume rather than a significant reduction of total cell number can be found in schizogenously formed aerenchymatous roots, including those in *Rumex* (Chapter 2). It cannot be stated however, that the one is more advantageous than the other, but it is possible that schizogenously formed aerenchyma, not resulting in a significant decrease in total cell number, needs cubic cell packing in order to quarantee sufficient pathways for oxygen diffusion. In addition, aerenchyma formation in *Rumex* roots is fully developed already within 250 µm from the root apex (P. Laan & G. Hendrickx, unpubl. results), but often not until 3-4 cm behind the root apex in lysigenous types (e.g. in rice roots, Armstrong 1979). This difference may have a large impact on the oxygen supply of the root apex, having a respiratory demand per unit volume 3-4 times higher than the rest of the root (P. Laan, unpubl. results): oxygen deficiency is more likely to occur in the apical zone of lysigenously formed aerenchyma types than in the schizogenous types, including *Rumex.*

Although it has been well-documented that ethylene is the main determinant for the formation of lysigenous aerenchyma (Drew *et al.* 1979; Kawase 1979), nothing is known of

the 'induction mechanism' of the honeycomb, schizogenously formed *Rumex* aerenchyma type. Indications for a regulation mechanism can be found in Chapter 7, as the outgrowth of new roots of *R. crispus* and *R. maritimus* appeared to be linked to a cessation of growth of the larger part of the primary roots. This observation can be explained by the fact that a growth reduction or cessation of growth of the primary roots leads to a lowered 'sink strength' for assimilates and thus to the formation of new roots. Alternatively, a hormonal 'feed-back' control, e.g. via cytokinins (which are synthesized in the growing root apices), may be proposed. Neither of these two explanations however, elucidate the fact that aerenchymatous, instead of non-aerenchymatous roots are formed in *R. maritimus* and *R. crispus,* nor do they clarify the differential responses of the *Rumex* species.

Implications of the balance between two root systems for tolerance of Rumex species to transient flooding

A number of plant species have been described that develop new roots in response to flooding (Drew 1983; Jackson & Drew 1984); in *Rumex* species new root formation is especially important for the flood-tolerance. The newly formed roots cannot simply be described as 'additional', since prolonged periods of flooding result in a very high contribution of new roots to the total root weight. This contribution can amount up to 70% of the total in hydroculture (Chapters 4 and 5), and in soil-culture the plants often have to rely completely on the new root system, since especially in the intermediate and flood-intolerant species the old roots die (Chapters 3, 6 and 7). The relative contribution of either of these two root systems to total root length will therefore ultimately determine the degree of flood-tolerance of the species. Oxygen supply is supposed to be the main determinant of these responses and a 'working model' was used in the initial phase of the project, described in this thesis (Fig. 2). This model summarizes the factors, supposed to determine the degree and the duration of root anaerobiosis, as well as the extent to which aerobiosis can be restored and applies especially to situations of transient flooding.

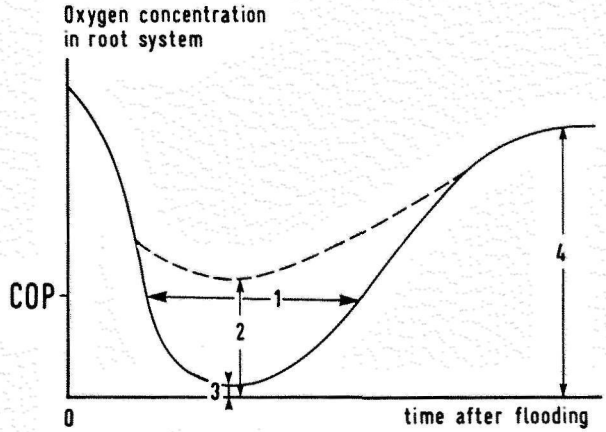


Figure 2 'Working model' for the course of oxygen concentration of the complete root system in *Rumex* species after the onset of flooding at t = 0; factors of importance for flood-tolerance indicated with numbers 1,2 and 3, representing the period of oxygen deficiency **(1)**, the degree of anaerobiosis in a root system of plants with **(2)** and without **(3)** resistance mechanisms, and the extent to which aerobiosis can be recovered by new root formation **(4)**

After the onset of flooding, the oxygen concentration in the soil, and consequently also in the root system of the plants, will decrease rapidly, although not to the same degree in all *Rumex* species because of 'internal aeration' (see Chapters 4 and 6). Therefore, the oxygen status of the root system will differ between the species (designated with no. 2 in Fig. 2). In their response to flooding the plants develop new roots at different rates and in different numbers (Chapter 2), which more or less restore the aerobic situation (Chapters 4 and 6). The rate with which these new roots develop, will determine the period of hypoxia or anoxia in the root system (no. 1 in Fig. 2) and their total number finally determines the extent to which the aerobic state is restored (no. 3 in Fig. 2). Applied to the three *Rumex* species under study, it was shown that the flood-tolerant *R. maritimus* and *R. crispus* develop new roots rapidly (3-4 days, Chapters 2 and 7), while new root formation in the flood-intolerant *R. thyrsiflorus* is slow (7-14 days). Also, the number of new roots formed was high in the former two species (Chapters 2 and 3), suggesting an efficient 'turn-over' from old to new root system, while in

R. thyrsiflorus only few new and slow growing roots develop (Chapters 2 and 7). These data indicate that *R. maritimus* will suffer least from oxygen depletion after flooding. The same seems to account for young *R. crispus* plants. With respect to the factors indicated in Figure 2, *R. thyrsiflorus* shows a highly inefficient response to flooding: a delayed development, and a low number and growth rate of new roots, in combination with the inability to develop aerenchyma (Chapter 2) result in an inability to aerate internally (Chapter 4). As a consequence, oxygen deficiency is likely to occur throughout the roots.

The importance of oxygen supply

For the *Rumex* species, oxygen availability seems to be of primary importance in determining flood-tolerance. High porosities throughout the plant result in low diffusive resistances, thereby facilitating internal longitudinal oxygen diffusion. Although water-logged and submerged situations are put forward here, this phenomenon extends to all situations, where oxygen supply is limited by diffusional impedance (Chapters 4 and 5; Table 1).

Internal aeration seems to be constitutive in *R. maritimus*, for it occurs under almost all circumstances (Table 1). Pre-aerenchymatous structures in primary roots and aerenchyma formation in the new roots serve this oxygen transport; in Chapter 4, I suggest that the degree of internal aeration is closely related to the number of newly formed roots, which may indicate the adaptive value of aerenchyma and new root formation in this species. It is nevertheless important not just to focus on the roots, but to consider whole plant porosity. As an annual species *R. maritimus* retains a high porosity throughout its life-cycle, in this way assuring internal aeration to take place until flowering. In contrast to this, *R. crispus* looses the ability of using internal aeration with age (Chapter 4). This was shown to be mainly due to the strong development of the woody tap-root, which, during its growth, increasingly forms a physical barrier for oxygen transport.

Table 1 Contribution of longitudinal internal oxygen transport to root respiration in *Rumex* species, subjected to different flooding levels (as a percentage of total root respiration at COPR (≡ 50-60 μM O_2) ± SE; data after Chapters 4 and 6)

Equivalent flooding level in field situation[1]	*R. maritimus*	*R. crispus*	*R. thyrsiflorus*
'Drainage'	29 ± 5	22 ± 4	<2
'Soil-flooding'	57 ± 8	39 ± 2	<5
'Submergence of shoot'			
Dark, 1/4 submerged	38 ± 10	nd[2]	nd
1/2 submerged	26 ± 5	nd	nd
3/4 submerged	15 ± 3	nd	nd
Totally submerged	1-5	nd	nd
Light, Totally submerged/0.5 mM CO_2	10 ± 1	nd	nd
Totally submerged/5 mM CO_2	50 ± 3	nd	nd

[1] Pretreatment of plants: 'drained', aerobically grown in hydroculture; 'soil-flooded' and 'submerged', anaerobically grown for one week in hydroculture, i.e. with new lateral roots developed; [2] nd, not determined.

In addition to longitudinal oxygen diffusion, radial oxygen diffusion can be important in situations of transient flooding, where oxygen is depleted from the soil and new roots have not yet developed, but also in compacted soils where oxygen diffusion is limited. Again, it was shown that the anatomy, diameter and respiratory demand of *R. maritimus* roots form the best combination to assure an efficient use of oxygen (Chapter 5). Notwithstanding its thin roots, the anatomy and respiratory activity of its root cells do not permit *R. thyrsiflorus* to cope efficiently with low oxygen supply.

'Avoidance' and 'Tolerance' mechanisms in relation to the differential life-histories of the species

The results of the experiments mentioned above indicate a low resistance of *Rumex thyrsiflorus* to flooding; nevertheless, the plants can survive a long period of soil-flooding and even total submergence (Chapter 6). And for *R. crispus*, none of the results, obtained in this thesis, point at a lower resistance of this species than that of

R. maritimus, despite of its intermediate position in the elevation zonation sequence. On the contrary, *R. crispus* plants survive a much longer period of total submergence than *R. maritimus* plants (Chapter 6).

These seemingly paradoxical observations may be reconciliated when viewed in the context of the age of the plants and of their different life-histories (Chapter 6). In addition, the term flood-intolerance can only be used for *R. thyrsiflorus* in relation to the other *Rumex* species under study; comparable experiments with *Senecio jacobaea* and *Anthriscus sylvestris* revealed that plants of these species die upon soil-flooding within 3 weeks, while merely growth reduction was found in *R. thyrsiflorus*.

The results obtained in this thesis predominantly pertain to young plants in an exponential phase of growth. The 'annual strategy' of *R. maritimus*, which is mainly characterized by a high growth rate, is apparently based upon continuous oxygen availability. Apparently, this strategy serves its function, but it is rigid and therefore risky, because it leads to the death of the plants when oxygen availability is not achieved (Chapter 6). On the other hand, the 'perennial strategy' of *R. thyrsiflorus* is not to adapt on the morphological level, but to reduce growth; the delay of the death of the plants then will depend mainly on the long-term sugar supply of the tap-root. The 'intermediate perennial strategy', exhibited by *R. crispus* is based on the use of internal aeration in the young life-phase and on metabolic adaptation in combination with dormancy in later stadia; it most probably provides the most flexible response towards different levels and periods of flooding.

These differences are illustrated by data on the potential use of anatomical and metabolical acclimation responses, summarized in Table 2 and comprised in terms of 'avoidance' and 'tolerance' resistance mechanisms in Table 3.

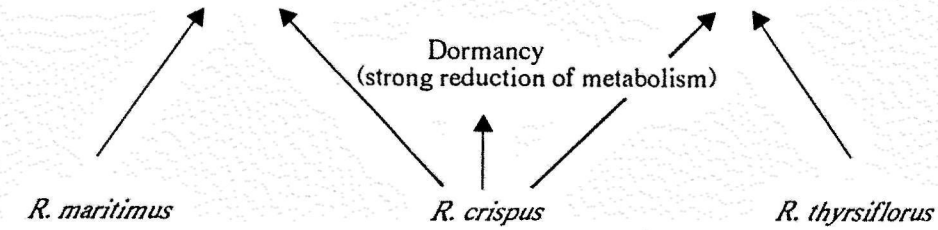
Table 2 Anatomical and metabolical acclimation of *Rumex* species to hypoxic and anoxic conditions (data represent percentage increase under anaerobic compared to aerobic, control conditions)

Adaptation	Aerenchyma formation[1]	'Pasteur effect'[2]	Induction of nitrate reductase[3]
Primary function	recovery of aerobic state	ATP production	reoxidation of NADH
R. thyrsiflorus	4%	90%	40%
R. crispus	21%	103%	202%
R. maritimus	21%	24%	-22%

[1] percentage increase in fractional root porosity (Chapter 2),
[2] percentage increase in CO_2-production with respect to the expected value of 0.33, after 3 d anaerobiosis (Chapter 9),
[3] percentage increase in nitrate reductase activity after 3 d anaerobiosis (Chapter 9).

Table 3 Proposed 'avoidance'- and 'tolerance'-mechanisms in *Rumex* species, upon transient flooding to different levels

"Avoidance"	*"Tolerance"*
Survival by avoiding the actual oxygen deficiency	Survival by tolerating oxygen deficiency
Rate of development of resistance mechanisms of primary importance	Duration and severity of stress of primary importance
Formation of new, aerenchymatous laterals (depending on flood-level, in combination with shoot elongation)	- 'Pasteur effect' - Use of nitrate as alternative electron acceptor



Future research

It appears desirable to elucidate some aspects of flood-tolerance not covered in this thesis. Among them, the physiological background ('trigger') and the real significance of 'dormancy' as resistance mechanism to stress conditions needs further investigation; the most attractive approach is to study the energetic costs and outputs. Furthermore, in addition to *in vitro* experiments, the number of experiments conducted with intact plants should be extended, as this seems to be the only ecophysiological approach that leads to a better understanding of the real significance of responses under field conditions.

An intriguing question that remains from Chapter 7 is to elucidate the 'trigger' mechanism of the *Rumex* aerenchyma, associated with new root formation. As mentioned earlier, a hormonal regulation via cytokinins may be speculated, because the outgrowth of new, aerenchymatous roots appeared to depend on the cessation of growth of the larger part of the primary roots. Such a mechanism might rather function in plants which are genotypically bound to the formation of a large number of roots with restricted root length and high root 'turnover' *(R. maritimus)*, than in plants with fewer and longer roots *(R. thyrsiflorus)*. Elucidating such a regulation mechanism will lead to a better understanding of the balance between genotypical variation and phenotypical plasticity.

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SAMENVATTING

Overstromingen en ondergelopen gronden, ten gevolge van inundatie, zijn van groot belang voor de wereldvoedselproduktie. Dit betreft vooral rijst, dat het overgrote deel van zuid en zuid-oost Azië van voedsel moet voorzien.

Het is reeds heel lang bekend dat bodems, die ten gevolge van excessieve regenval of inundatie met water verzadigd raken, nadelige eigenschappen hebben op de groei van planten (*Flavius Josephus,* AD 100). Veel later ontdekte men welke problemen een rol spelen bij inundatie. Primair is dat steeds zuurstofgebrek; ten gevolge van overstroming wordt de uitwisseling van gassen tussen de atmosfeer en de bodem min of meer afgesloten, daar de diffusie van gassen in een waterig medium ca. 10.000 maal zo langzaam verloopt als in lucht. Plantewortels, maar in veel sterkere mate bodemmicro-organismen, verbruiken dermate veel zuurstof dat al binnen enkele uren een anaerobe situatie in de bodem kan ontstaan. Een bijkomend probleem voor de plant is dat, wanneer alle zuurstof uit de bodem verdwenen is, de micro-organismen andere terminale acceptoren voor hun electronentransportketen gaan gebruiken. Dit leidt tot een zogenoemde bodemreductie, waarbij vele essentiële macro-nutriënten verdwijnen en potentieel toxische stoffen verschijnen. Bovendien wordt zo'n bodem zuurstofbehoeftig: alle zuurstof die uit de plant of door diffusie uit de lucht verschijnt wordt onmiddellijk verbruikt door de micro-organismen.

Planten die op dergelijke bodems voorkomen, moeten dus aanpassingen getroffen hebben om de bovengenoemde problemen het hoofd te kunnen bieden. Omdat de cellen van alle plantesoorten, ook van die soorten die onder continue geïnundeerde omstandigheden voorkomen, obligaat aeroob zijn, zullen de aanpassingen die leiden tot een grote resistentie voornamelijk gezocht moeten worden in een herstel van de zuurstofvoorziening van het wortelstelsel. Kortdurende periodes van zuurstofgebrek echter, kunnen ook overleefd worden m.b.v. aanpassingen op metabool niveau.

In dit proefschrift worden verschillende aanpassingsmechanismen beschreven voor een aantal zuring *(Rumex)* -soorten. Een groot aantal van deze zuringsoorten komt voor in het rivierengebied rondom Nijmegen. Gedurende de laatste 10-15 jaar worden de uiterwaarden in het rivierengebied ten gevolge van civiel- en cultuurtechnische verbeteringen in de bovenloop van de Rijn onregelmatig overstroomd in het groeiseizoen van de planten. Deze overstromingen zijn geheel onvoorspelbaar wat betreft hun tijdstip, hoogte, duur en frequentie, waardoor de planten in verschillende levensstadia en met verschillende inundatiehoogte geconfronteerd worden: van kortdurende inundatie van de bodem tot langdurige periodes van totale onderdompeling van de plant. Mede hierdoor is een zonering van zuringsoorten ontstaan in de uiterwaarden. Bij de aanvang van dit project werd deze zonering voornamelijk toegeschreven aan verschillen in de resistenties van de soorten tegen inundatie. Het oecofysiologisch onderzoek werd, vanwege de complexiteit van het systeem, beperkt tot experimenten met jonge planten, die zich in een exponentieel groeistadium bevonden; bovendien werd de dynamiek van het rivieren-oecosysteem gereduceerd tot een tweetal inundatieniveaus: bodeminundatie en totale onderdompeling van de plant.

In het begin was het onderzoek vooral gericht op een inventarisatie van verschillen in morfologische en anatomische responsen van de soorten op bodeminundatie, beschreven in Hoofdstuk 2. Hieruit bleek dat de gevonden zonering van de zuringsoorten sterk gecorreleerd was met de mate waarin nieuwe wortels werden gevormd: soorten die laag in de zonering voorkwamen bleken meer en sneller nieuwe wortels te vormen dan soorten die hoger gelegen habitats bezetten. De verklaring voor deze verschillen bleek heel duidelijk uit anatomisch onderzoek. Nieuwe wortels van de 'tolerante' soorten bleken in staat te zijn om een uitgebreid stelsel van longitudinaal verbonden luchtholtes (aerenchym) te ontwikkelen.

De functionele betekenis van de vorming van een nieuw, poreus wortelstelsel wordt beschreven in de Hoofdstukken 3 en 4. Door de vorming van een nieuw, poreus wortelstelsel wordt het interne transport van zuurstof van de spruit naar de wortel vergemakkelijkt en door verlies van zuurstof kan bodemoxydatie optreden. Tolerante zuringen hadden een hoog

radiaal zuurstofverlies (ROL, Hoofdstuk 3), waardoor grote delen van de bodem geoxideerd kunnen worden. Dit heeft als positief gevolg dat potentiële toxines, zoals gereduceerd ijzer, geïmmobiliseerd worden; bodemoxydatie heeft tevens tot gevolg dat de negatieve effecten van inundatie op de beschikbaarheid van nutriënten tegengegaan worden, waardoor de biomassaproduktie van de plant onder bodem-geïnundeerde omstandigheden gehandhaafd kan blijven.

Doordat de vorming van aerenchym de diffusieweerstand voor gastransport verlaagt, is de aanvoer van zuurstof naar het wortelstelsel verzekerd. Dit bleek tot gevolg te hebben dat de worteladem haling van de tolerante zuringsoorten voor een groot deel verzorgd werd door zuurstof, afkomstig uit de lucht ('interne aeratie', Hoofdstuk 4). Hierdoor kunnen planten onder geïnundeerde of anderszins anaerobe omstandigheden de aerobe ademhaling handhaven. Uit het feit dat ook planten zonder nieuwe, aerenchymatische wortels 'interne aeratie' gebruiken voor de worteladem haling bleek dat vooral de totale diffusieweerstand van de plant bepalend is voor de mate waarin dit proces optreedt; het belang van interne aeratie is daarom niet slechts beperkt tot anaerobe situaties, maar lijkt meer algemene geldigheid te hebben.

In Hoofdstuk 5 wordt beschreven of er naast verschillen in longitudinaal, ook verschillen in radiaal zuurstoftransport bestonden tussen aeroob opgekweekte zuringsoorten.
Dit kan van groot belang zijn bij dalende zuurstofspanningen in de bodem ten gevolge van tijdelijke overstromingen of in compacte, niet overstroomde bodems. Het bleek dat verschillen in ademhalingskarakteristieken tussen de soorten volledig toegeschreven konden worden aan verschillen in wortelanatomie en niet aan biochemische verschillen van de ademhaling. Ook hier bleken de tolerante soorten het meest efficiënt zuurstof op te kunnen nemen bij lage zuurstofspanningen in de omgeving.

De verschillen in overstromingstolerantie en de aanpassingsmogelijkheden van de planten komen het meest duidelijk tot uiting onder extreme omstandigheden. In Hoofdstuk 6 worden de responsen van de planten onder bodem-geïnundeerde en totaal geinundeerde

omstandigheden beschreven. Totale inundatie leidde tot de dood van de planten en dit bleek slechts voorkomen te kunnen worden door een vroegtijdige 'dormancy', het gebruik van onderwater-fotosynthese of een stengelstrekking waardoor het luchtcontact hersteld wordt. Vooral verschillen in levenscyclus-strategieën van de soorten worden benadrukt.

In Hoofdstuk 7 worden groeikarakteristieken van het voor de zuringsoorten zo kenmerkende nieuwe wortelstelsel vergeleken met het reeds bestaande wortelstelsel. Hierbij staan de efficiëntie van de 'turnover' van het oude naar het nieuwe wortelstelsel en de mogelijk achterliggende regulatiemechanismen centraal.

In een hydroculture-experiment bleek het gecombineerde effect van anaerobie en hoge concentraties gereduceerd ijzer negatieve effecten op de wortelgroei van de intolerante zuringsoort te veroorzaken (Hoofdstuk 8). Dit was vooral het gevolg van een slechte zuurstofvoorziening van het wortelstelsel. Vergelijkbare omstandigheden (bodeminundatie en hoge ijzerconcentraties) in een kasproef lieten echter zien dat de biomassaproduktie niet nadelig werd beïnvloed door naast bodeminundatie, een extra stressfactor (vrij ijzer), aan te brengen. Ofschoon verschillende vormen van ijzertoxiciteit op zowel blad als bladsteel te zien waren, leidde dit niet tot een verminderde biomassaproduktie, hetgeen kon worden toegeschreven aan de mogelijkheid om door bodemoxydatie aan de negatieve gevolgen te kunnen ontsnappen.

Tenslotte worden de mogelijkheden en het belang van metabole aanpassingen voor de zuringsoorten besproken (Hoofdstuk 9). Onder hypoxische of anoxische omstandigheden van de wortelcellen werd in alle soorten de alcoholfermentatie gebruikt om de negatieve gevolgen van zuurstofgebrek te kunnen compenseren. Naast de inductie van de belangrijkste enzymen in de fermentatiereacties, t.w. alcohol dehydrogenase en pyruvaat decarboxylase, bleek dat ook nitraatreductase een functie kon vervullen in de metabole adaptatie van een aantal zuringsoorten.

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CURRICULUM VITAE

Peter Laan werd op 1 juni 1957 in Hoorn (NH) geboren. Na het behalen van zijn HAVO diploma in 1975 aan de SG Werenfridus te Hoorn, werd de Lerarenopleiding VL/VU te Amsterdam doorlopen, waar het hoofdvak Biologie en het bijvak Scheikunde was. In februari 1980 werd aan de Vrije Universiteit te Amsterdam en later ook aan de Universiteit van Amsterdam de studie Biologie voortgezet. Het doctoraalprogramma omvatte twee hoofd- en twee bijvakken. Het eerste deel van het hoofdvak werd op de afdeling Experimentele Plantenoecologie van de VU uitgevoerd (begeleiding: dr. J. Rozema, prof. dr. W.H.O. Ernst), het aansluitende tweede deel op de vakgroep Plantenfysiologie van de UvA (begeleiding: drs. P.C. Sijmons, dr. H.F. Bienfait). Het eerste bijvak werd op de vakgroep Electronenmicroscopie en Moleculaire Cytologie onder begeleiding van dr. C.L. Woldringh aan de UvA uitgevoerd, het tweede op de afdeling Dieroecologie van de VU (begeleiding: dr. A. Kessler, prof. dr. E.N.G. Joose-van Damme). Naast de reeds behaalde 2e graads lesbevoegdheid in de Biologie en de Scheikunde, werd als onderdeel van het doctoraalprogramma de 1e graads lesbevoegdheid bij de vakgroep Vakdidactiek van de VU behaald.

Naast de doctoraalstudie was hij van februari tot juni 1983 werkzaam als docent Biologie aan de Pedagogische Academie te Alkmaar.

Vanaf september 1985 werd een promotieonderzoek aan de Katholieke Universiteit te Nijmegen uitgevoerd onder supervisie van prof. dr. C.W.P.M. Blom en prof. dr. J.T. Lambers (Rijksuniversiteit Utrecht). Naast dit promotieonderzoek werd de cursus Oecofysiologie t.b.v. 3e jaars studenten opgezet en uitgevoerd. Hierin waren een aantal gastdocenten betrokken. Verder werd een aantal excursies t.b.v. de 2e jaars en de doctoraalstudenten georganiseerd.

Per 1 maart 1990 is hij werkzaam als post-doc bij prof. dr. H. Marschner (Universiteit Hohenheim, Duitsland).

STELLINGEN

1. De belangrijkste factor, die de mate van overstromingstolerantie bepaalt, is de mogelijkheid om het zuurstoftekort van het wortelstelsel te voorkomen of op te heffen d.m.v. intern longitudinaal zuurstoftransport
   (ap Rees *et al.* 1987; Brändle 1990; dit proefschrift).
2. Metabole aanpassingen zijn slechts van beperkt belang voor de overstromingstolerantie van planten en moeten meer beschouwd worden als primaire stress reactie dan als een adaptatie *sensu stricto* (dit proefschrift).
3. Als niet-aerenchymatische celconfiguratie is de kubische celopbouw het best toegesneden op een optimale zuurstofvoorziening van de wortels (Justin & Armstrong 1987).
4. Voor schizogeen aerenchym, waarbij geen sterke reductie van het totale aantal cellen plaatsvindt, is de kubische celconfiguratie de beste verzekering om aan de zuurstof-behoefte van de wortelcellen te voldoen (dit proefschrift).
5. Het aantal publicaties over een onderwerp zegt niets over het belang van dat onderwerp
6. Te vaak nog worden resultaten, verkregen uit experimenten met plantendelen, geïnterpreteerd naar de situatie voor intacte planten.
7. Wanneer de ontwikkeling in de Nederlandse verkeerssituatie zich voortzet volgens dezelfde trend als de afgelopen 10 jaar, zullen fietsers moeten wennen aan het idee straks rond te rijden met valhelm en zwaailicht.
8. Diegene die nooit een pipet vasthoudt, kan deze ook niet laten vallen
9. Een 'avoidance'-mechanisme is zowel voor de hier onderzochte *Rumex* soorten, alsook voor de mens, op langere termijn de beste resistentie tegen stress.

10. Wetenschap behoort kinder-Spel te zijn.
11. In tegenstelling tot de situatie in grote Europese steden als Parijs, Londen of Praag, wordt op de roltrappen van Utrecht en Amsterdam Centraal Station geen ruimte gelaten voor mensen die willen passeren; dit illustreert een intolerante houding van Nederlanders jegens mensen die iets willen.
12. De kwaliteit van het universitair onderwijs gaat niet achteruit door de vermeende studieduurverkorting ten gevolge van de twee-fasen structuur, maar vooral door een gebrek aan onderwijskundig en vaktechnisch goed opgeleid onderwijzend personeel.

Beek-Ubbergen, 18 Juni 1990

Peter Laan